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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 9/02, 15/53, 15/63, 1/21, 15/09, 15/10, C12Q 1/68, 1/26 — A1

(11) International Publication Number: WO 97/48793

(43) International Publication Date: 24 December 1997 (24.12.97)

(21) International Application Number: PCT/US97/10644

(22) International Filing Date: 20 June 1997 (20.06.97)

(30) Priority Data:
60/022,086 21 June 1996 (21.06.96) US

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(81) Designated States: AU, CA, CN, JP, MX, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: PLANT STEROL REDUCTASES AND USES THEREOF

(57) Abstract

Disclosed are plant sterol biosynthetic enzymes, genes, and their uses.

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PLANT STEROL REDUCTASES AND USES THEREOF

Background of the Invention

This application relates to plant sterol biosynthetic enzymes, genes, and their uses.

Plant sterols belong to a large group of secondary compounds known as terpenes or isoprenoids. Sterol biosynthesis in plants generally involves a series of different enzymatic steps in the isoprenoid pathway that result in the formation of a variety of sterol end products (Benveniste *Ann. Rev. Biochem.* 37:275, 1986). Although such sterol compounds have been identified in higher plants, their function in plant growth and development is poorly understood.

One such plant sterol, brassinolide, that belongs to a class of sterols referred to as brassinosterioids (BR), was first discovered in the pollen of *Brassica napus* (Grove et. al., *Nature* 281: 216, 1979). Brassinosteroids are growth-promoting natural products having structural similarities to animal steroid hormones. The wide distribution of brassinosteroids in the plant kingdom, their effect on cell proliferation and elongation, and their interactions with other plant hormones (e.g., cytokinins), have indicated that these compounds are plant-growth regulators. Brassinosteroids are thought to promote hypocotyl elongation, leaf unrolling, and xylem differentiation. In addition, such compounds are also believed to be involved in de-etiolation of cotyledons, root elongation, radial growth, and anthocyanin formation.

The function of plant sterol growth regulators, such as BR, in relationship to other classes of plant growth regulators such as auxin, gibberellin, abscisic acid, and cytokinin, during plant development also needs to be evaluated. For example, the growth regulator, cytokinin, is known to affect a variety of developmental processes including photomorphogenesis, chloroplast biogenesis and maintenance, apical

dominance, and senescence. In addition, this growth regulator is thought to antagonize BR's ability to promote hypocotyl elongation and cotyledon de-etiolation.

Summary of the Invention

In general, the invention features a substantially pure plant C-14 sterol reductase polypeptide. Preferably, the C-14 sterol reductase polypeptide includes an amino acid sequence substantially identical to the sequence shown in Fig. 14 (SEQ ID NO: 1); and is from a dicot (for example, a crucifer or a solanaceous plant), monocot, gymnosperm, or an alga.

In related aspects, the invention features purified DNA that includes a sequence encoding a C-14 sterol reductase polypeptide (for example, a sequence substantially identical to the DNA sequence shown in Fig. 14; SEQ ID NO: 2; or a DNA sequence that encodes a C-14 sterol reductase polypeptide which has an amino acid sequence substantially identical to that shown in Fig. 14; SEQ ID NO: 1). The invention also features a vector and a cell, each of which includes purified DNA encoding a C-14 sterol reductase polypeptide; and a method of producing a recombinant C-14 sterol reductase polypeptide involving providing a cell (for example, a plant cell) transformed with purified DNA encoding a C-14 sterol reductase polypeptide positioned for expression in the cell, culturing the transformed cell under conditions for expressing the DNA, and isolating the recombinant C-14 sterol reductase polypeptide. The invention further features recombinant C-14 sterol reductase produced by such expression of a purified DNA, and an isolated antibody that specifically recognizes and binds a plant C-14 sterol reductase polypeptide.

In addition, the invention features nucleotide sequences that hybridize to a C-14 sterol reductase gene (including the coding sequence of such a gene and its complement) and that encode a C-14 sterol reductase polypeptide. Furthermore, the invention includes oligonucleotide probes that detect a C-14 sterol reductase gene or functional equivalents thereof in a plant (for example, dicots (such as solanaceous and

cruciferous plants), monocots, gymnosperms, and algae). Such probes are useful to isolate DNA sequences that encode C-14 sterol reductases from other plants. In one particular example, oligonucleotides may be designed based on a C-14 sterol reductase sequence disclosed herein and used as hybridization probes or as primers in polymerase chain reactions (PCR). Conserved regions in the C-14 sterol reductase gene are useful in the design of such primers to facilitate the recovery of C-14 sterol reductases from other related and unrelated plants.

In yet other related aspects, the invention features a transgenic plant (or seeds or cells thereof) containing DNA encoding a C-14 sterol reductase polypeptide integrated into the genome of the plant, where the DNA is expressed in the transgenic plant, resulting in the production of a C-14 sterol reductase polypeptide.

In still another aspect, the invention features a method for reducing the level of a plant C-14 sterol reductase polypeptide in a transgenic plant cell. This method generally involves expressing in the transgenic plant cell an antisense C-14 sterol reductase polypeptide nucleic acid sequence. In general, such an antisense C-14 sterol reductase nucleic acid sequence is encoded by a transgene integrated into the genome of the transgenic plant cell and is based on the nucleotide sequence that is shown in Fig. 14 (SEQ ID NO: 2) or Fig. 15. (SEQ ID NO: 3). In preferred embodiments, the plant cell expressing an antisense C-14 sterol reductase nucleic acid sequence is a dicot (for example, crucifer), monocot, gymnosperm, or algal cell. In yet other preferred embodiments, the method involves growing a transgenic plant from the transgenic plant cell, whereby the level of the C-14 sterol reductase polypeptide is reduced in the transgenic plant.

In other related aspects, the invention features a plant cell expressing an antisense C-14 sterol reductase nucleic acid sequence and a plant expression vector that includes an antisense C-14 sterol reductase nucleic acid sequence, where the antisense sequence is operably linked to an expression control region.

In another aspect, the invention features a method for increasing the level of a C-14 sterol reductase in a transgenic plant cell. This method involves expressing in the transgenic plant cell a C-14 sterol reductase polypeptide nucleic acid sequence. Preferably, the method utilizes a C-14 sterol reductase nucleic acid sequence that is substantially identical to the nucleotide sequence that is shown Fig. 14 (SEQ ID NO: 2). In preferred embodiments, the plant cell expressing a C-14 sterol reductase polypeptide nucleic acid sequence is a dicot (for example, a crucifer), monocot, gymnosperm, or algal cell.

In another aspect, the invention features a transgenic plant having a knockout mutation in DNA encoding a plant C-14 sterol reductase polypeptide. Such knockout genes are constructed according to conventional methods (e.g., Lee et al. *Plant Cell* 2: 415, 1990; Miao and Lam, *Plant J.* 7: 359, 1995).

By "plant C-14 sterol reductase" is meant an amino acid sequence that catalyzes the reduction of any sterol precursor having a C14=C15 double bond, for example, as described by Benveniste, *Annu. Rev. Biochem.* 37: 275, 1986. Preferably, such a polypeptide has an amino acid sequence which is at least 30%, preferably 40%, and most preferably 50% or even 80-95% identical to the amino acid sequence of the C-14 sterol reductase polypeptide shown in Fig. 14 (SEQ ID NO: 1). The length of comparison of amino acid sequences will generally be at least 16 amino acids, preferably at least 20 amino acids, more preferably at least 25 amino acids, and most preferably at least 35 amino acids.

By "polypeptide" or "protein" is meant any chain of amino acids, regardless of length or post-translational modification (for example, glycosylation or phosphorylation).

By a "substantially identical" polypeptide sequence is meant an amino acid sequence that differs only by conservative amino acid substitutions, for example, substitution of one amino acid for another of the same class (e.g., valine for glycine, arginine for lysine, etc.) or by one or more non-conservative substitutions, deletions,

or insertions, located at positions of the amino acid sequence that do not destroy the function of the polypeptide (assayed, for example, as described herein).

Sequence identity is typically measured using sequence analysis software (for example, Sequence Analysis Software Package of the Genetics Computer Group (University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705), BLAST, or PILEUP/PRETTYBOX programs). Such software matches sequences by assigning degrees of homology to various substitutions, deletions, and other modifications.

By "substantially pure polypeptide" is meant a polypeptide preparation that is at least 60% by weight (dry weight) the compound of interest, for example, the C-14 sterol reductase polypeptide or C-14 sterol reductase-specific antibody. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight the compound of interest. Purity can be measured by any appropriate method, for example, column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

By "purified DNA" is meant DNA that is not immediately contiguous with both of the coding sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally-occurring genome of the organism from which it is derived. The term therefore includes, for example, a recombinant DNA that is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote, or that exists as a separate molecule (for example, a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences. It also includes a recombinant DNA that is part of a hybrid gene encoding one or more additional amino acids.

By a "substantially identical" nucleic acid is meant a nucleic acid sequence that encodes a polypeptide differing only by conservative amino acid substitutions, for example, substitution of one amino acid for another of the same class (e.g., valine for

glycine, arginine for lysine, etc.) or by one or more non-conservative substitutions, deletions, or insertions, located at positions of the amino acid sequence that do not destroy the function of the polypeptide (assayed, for example, as described herein). Again, the encoded sequence is at least 30%, more preferably 40%, and most preferably 50%, or even 80 to 95% identical at the amino acid level to the sequence of Fig. 14 (SEQ ID NO: 1). Thus, when nucleic acid sequences are compared, a "substantially identical" nucleic acid sequence is one which is at least 30%, more preferably 40%, and most preferably 50%, or even 80 to 95% identical to the sequence of Fig. 14 (SEQ ID NO: 2). The length of nucleic acid sequence comparison will generally be at least 30 nucleotides, preferably at least 60 nucleotides, more preferably at least 75 nucleotides, and most preferably 110 nucleotides. Again, identity is typically measured using sequence analysis software (for example, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705).

By "isolated antibody" is meant antibody that is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, antibody.

By "specifically binds" is meant an antibody that recognizes and binds a C-14 sterol reductase polypeptide but which does not substantially recognize and bind other molecules in a sample (e.g., a biological sample) which naturally includes a C-14 sterol reductase. An antibody which "specifically binds" a C-14 sterol reductase is sufficient to detect a C-14 sterol reductase product in such a biological sample using one or more of the standard immunological techniques available to those in the art (for example, Western blotting or immunoprecipitation).

By "an antisense C-14 sterol reductase sequence" is meant a nucleotide sequence that is complementary to a plant C-14 sterol reductase messenger RNA. In general, such an antisense sequence will usually be at least 15 nucleotides, preferably

about 15-200 nucleotides, and more preferably 200-2,000 nucleotides in length. The antisense sequence may be complementary to all or a portion of the plant C-14 sterol reductase mRNA nucleotide sequence, and, as appreciated by those skilled in the art, the particular site or sites to which the antisense sequence binds as well as the length of the antisense sequence will vary, depending upon the degree of inhibition desired and the uniqueness of the antisense sequence. By binding to the appropriate target sequence, an RNA-RNA, DNA-DNA, or RNA-DNA duplex is formed. A transcriptional construct expressing a plant C-14 sterol reductase antisense nucleotide sequence includes, in the direction of transcription, a promoter, the sequence coding for the antisense RNA on the sense strand, and a transcriptional termination region. Antisense C-14 sterol reductase sequences may be constructed and expressed as described herein or as described, for example, in van der Krol et al., *Gene* 72: 45, 1988; Rodermel et al., *Cell* 55: 673, 1988; Mol et al., *FEBS Lett.* 268: 427, 1990; Weigel and Nilsson, *Nature* 377: 495, 1995; Cheung et al., *Cell* 82, 383, 1995; and U.S. Pat. No. 5,107,065. In addition, C-14 sterol reductase antisense sequences are useful for the formation of triple helices, where the antisense sequence is bound to a DNA duplex. By binding to the target nucleic acid, C-14 sterol reductase antisense sequences can inhibit the function of the target nucleic acid. This results, for example, in the blocking of transcription, processing of poly A+ addition, replication, translation, or promoting inhibitory mechanisms of the cell, such as RNA degradation. The triple helix-forming and antisense C-14 sterol reductase sequences are useful for selectively suppressing certain cellular functions that are associated with C-14 sterol reductase activity.

By a "transformed cell" is meant a cell into which (or into an ancestor of which) has been introduced, by means of recombinant DNA techniques, a DNA molecule encoding (as used herein) a C-14 sterol reductase polypeptide (for example, a substantially identical DNA encoding the C-14 sterol reductase shown in Fig. 14 (SEQ ID NO: 2)).

By "positioned for expression" is meant that the DNA molecule is positioned adjacent to a DNA sequence which directs transcription and translation of the sequence (for example, facilitates the production of, for example, a plant C-14 sterol reductase polypeptide such as the amino acid sequence shown in Fig. 14 (SEQ ID NO: 1)), or an RNA molecule (for example, an antisense RNA).

By "promoter" is meant a minimal sequence sufficient to direct transcription. Included in the invention are promoter elements that are sufficient to render promoter-dependent gene expression controllable for cell-, tissue-, or organ-specific gene expression, or elements that are inducible by external signals or agents (for example, light-, pathogen-, wound-, stress-, or hormone-inducible elements); such elements may be located in the 5' or 3' regions of the native gene or engineered into a transgene construct.

By "operably linked" is meant that a gene and a regulatory sequence(s) are connected in such a way as to permit gene expression when the appropriate molecules (for example, transcriptional activator proteins) are bound to the regulatory sequence(s).

By "crucifer" is meant any plant that is classified within the Cruciferae family as commonly described in, e.g., Gray's Manual of Botany American Book Company, N.Y., 1950; *Hortus Third: A Concise Dictionary of Plants Cultivated in the U.S. and Canada*, Macmillan, 1976; or Simmons, N.W., *Evolution of Crop Plants*, 1986. The Cruciferae include many agricultural crops, including, but not limited to, broccoli, cabbage, brussel sprouts, rapeseed, kale, Chinese kale, cauliflower, horseradish, and *Arabidopsis*.

By "plant cell" is meant any self-propagating cell bounded by a semi-permeable membrane and containing a plastid. Such a cell also requires a cell wall if further propagation is desired. Plant cell, as used herein includes, without limitation, algae, cyanobacteria, seeds, suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen, and microspores.

By "transgene" is meant any piece of DNA that is inserted by artifice into a cell, and becomes part of the genome of the organism which develops from that cell. Such a transgene may include a gene which is partly or entirely heterologous (i.e., foreign) to the transgenic organism, or may represent a gene homologous to an endogenous gene of the organism.

By "transgenic" is meant any cell that includes a DNA sequence which is inserted by artifice into a cell and becomes part of the genome of the organism which develops from that cell. As used herein, the transgenic organisms are generally transgenic plants and the DNA (transgene) is inserted by artifice into the nuclear or plastidic genomes.

Other features and advantages of the invention will be apparent from the following detailed description thereof, and from the claims.

Detailed Description

The drawings will first be described.

Drawings

Figs. 1A-1B are photographs showing that the *ell* mutant morphology was phenocopied by treating wild-type seedlings with 30 μM of dimethylallylamino-purine (2ip), a synthetic cytokinin, in the dark **(Fig. 1A)** or light **(Fig. 1B)**. From left to right in both **Figs. 1A-1B**: wild-type plant; wild-type plant + 2ip; *ell*; and *ell* + 2ip.

Fig. 2 is a photograph illustrating the constitutive photomorphogenesis of *ell* seedling development in the dark. Wild-type (left) and *ell* (right) seedlings were grown in the dark for twenty-one days on Murashige-Skoog (MS) plates containing two percent sucrose.

Figs. 3A-3B are photographs showing that the rosette leaves of the *ell* plant **(Fig. 3B)** are darker green in color that those of the wild-type plant **(Fig. 3A)**.

Fig. 4 is a photograph illustrating that an *ell* mutant has reduced apical dominance in comparison to a wild-type plant. Six-week-old wild-type (left) and ten-week-old *ell* (right) plants were grown in the greenhouse.

Fig. 5 is a photograph showing that *ell* mutants (right) exhibit irregular, thickened cotyledons and hypocotyls, and reduced cotyledon petioles compared to wild-type plants (left).

Figs. 6A-6B are photographs showing abnormal flower development in the *ell* mutant. **Fig. 6A** shows, from left to right, that the sepal, petal, stamen, and carpel are shorter in *ell* (lower row) than wild-type (upper row) plants. **Fig. 6B** shows, from left to right, the top and side view of wild-type (left) and *ell* (right) flowers.

Figs. 7A-7F are photographs showing embryo development in *ell* and wild-type plants. Wild-type and *ell* plants are shown in the left and right of each photograph, respectively. **Fig. 7A** shows *ell* embryo development at the 32- to 64-cell stage, and **Fig. 7B** shows that, when wild-type embryos have reached the heart stage, *ell* embryos are only at the globular stage. As shown in **Fig. 7C** and **Fig. 7D**, when the wild-type embryo reached the torpedo stage, the *ell* mutant embryo was at the heart stage. **Fig. 7E** shows that apical hooks were not formed in *ell* embryos. And

Fig. 7F shows that *ell* seeds desiccated without completing the late stages of embryogenesis.

Figs. 8A-8B are photographs of dry seeds from wild-type **(Fig. 8A)** and *ell* plants **(Fig. 8B)**. Reduced seed size, wrinkled seed coat, and precocious germination were observed in *ell* seeds.

Figs. 9A-9F are photographs showing the supernumerary cotyledons that were observed in the *ell* mutant, including one **(Fig. 9A)**, two **(Fig. 9B)**, three **(Fig. 9C)**, four **(Fig. 9D)**, five **(Fig. 9E)**, and more than six cotyledons **(Fig. 9F)**.

Figs. 10A-10B are illustrations showing various aspects of the molecular characterization of the *Arabidopsis thaliana* C-14 sterol reductase gene. **Fig. 10A** is a schematic illustration showing the position of a T-DNA insertion into chromosome 3 of *Arabidopsis*, approximately forty base pairs upstream of the *ELL* gene, and the exon-intron structure of the C-14 sterol reductase gene.

Fig. 10B is a schematic illustration showing the map position of *ELL* on chromosome 3.

Fig. 11 is a schematic illustration showing a comparison of the predicated *ELL* amino acid sequence (designated Ath; SEQ ID NO: 1) with C-14 sterol reductase of *Saccharomyces cerevisiae* (Erg24) and *Schizosaccharomyces pombe* (Pombe), and C-24 sterol reductase of *Sz. pombe* (Sts1) and *S. cerevisiae* (Ygl022).

Fig. 12 is a schematic illustration showing that the predicted *ELL* amino acid sequence (designated Ath; SEQ ID NO: 1) shares homology to human and chicken lamin B receptor.

Figs. 13A-13B are photographs showing that the *ell* phenotype was not corrected by exogenous feeding of brassinolide (1 μM) in either dark **(Fig. 13A)** or light **(Fig. 13B)**. From left to right in **Figs. 13A-13B**: wild-type; *ell*; wild-type + brassinolide; and *ell* + brassinolide.

Fig. 14 is a schematic illustration showing the nucleotide sequence of an *Arabidopsis* C-14 sterol reductase (SEQ ID NO: 2) and its deduced amino acid sequence polypeptide (SEQ ID NO: 1).

Fig. 15 is a schematic illustration showing the genomic nucleotide sequence of an *Arabidopsis* C-14 sterol reductase polypeptide (SEQ ID NO: 3).

Fig. 16 is a schematic illustration showing the sequence comparison between the genomic nucleotide sequence (SEQ ID NO: 3) and cDNA sequences (SEQ ID NO: 2) of an *Arabidopsis* C-14 sterol reductase.

There now follows a description of an *Arabidopsis* mutant, *ell* (extra long life), that displays a life span that is at least three times greater than wild-type plants. The *ell* mutant was isolated by T-DNA tagging methods and was shown to encode a novel C-14 sterol reductase. This example is provided for the purpose of illustrating the invention, and should not be construed as limiting.

Identification and Developmental Effects of the *ell* Mutation By screening for mutants displaying BR deficiency or constitutive cytokinin activity, a recessive mutation causing pleiotropic developmental effects was identified according to conventional methods in an *Arabidopsis* T-DNA insertional mutant collection (Feldmann, *Plant J.* 1:71, 1991; Errampalli et al., *Plant Cell* 3: 149, 1991). This mutant, termed "ell", was found to have a number of developmental abnormalities.

For example, unlike wild-type plants, *ell* mutants displayed constitutive light-morphogenesis (Fig. 2), similar to the *Arabidopsis det2* (Chory et al., *Plant Cell* 3: 445, 1991) and *cpd* (Szekeres et al., *Cell* 85: 171, 1996) mutants. In addition, compared to wild-type plants, *ell* plants had darker green rosette leaves (Figs. 3A-3B), reduced apical dominance (Fig. 4), stunted hairy roots, and irregular hypocotyl and cotyledons (Fig. 5). Furthermore, as shown in Figs. 6A-6B, the *ell* mutant showed reduced and ruffled sepals and petals. The *ell* mutant also showed delayed and altered

embryo development (Figs. 7A-7F) and was found to have reduced fertility, producing wrinkled seeds that precociously germinated (Figs. 8A-8B). In addition, the various phenotypes of *ell* overlapped with *amp-1* (*pt1*) (Chaudhury et. al., *Plant J.* 4: 907, 1993) and *häuptling* (Jürgens et al., *Ann. Rev. Genet.* 28: 351, 1994), including supernumerary cotyledons (Figs. 9A-9F).

Finally, as shown in Figs. 1A-1B, the morphology of the T-DNA-tagged *ell* mutant seedlings was phenocopied by treating wild-type seedlings with 30 μM dimethylallylamine purine (2ip), a synthetic cytokinin.

Despite having a number of developmental abnormalities, *ell* mutants were found to have a life span that was at least three times greater than wild-type plants.

Genetic Analysis and Molecular Cloning of *ELL*

Standard segregation analysis indicated that *ell* is a recessive mutation. The T2 population of the transgenic line carrying the *ell* mutant showed a 3:1 Mendelian segregation of the T-DNA using kanamycin resistance (kan^r) as a selectable marker. Of the kan^r plants, thirty-three percent showed the *ell* phenotype, indicating that the *ell* mutation was recessive. A T3 population was then generated from selfed T2 kan^r plants having the wild-type phenotype, and the kan^r marker showed a 3:1 segregation. Of the seventy-five percent displaying kan^r, twenty-five percent showed the *ell* phenotype. Because *ell* homozygous plants were found to be either lethal or sterile, T2 heterozygous *ell* plants were subsequently backcrossed to wild-type plants for additional segregation analysis. The resulting F1 population from this backcross showed a 1:1 segregation of the kan^r marker; no plants were observed having the *ell* phenotype. The F1 kan^r individuals of the backcross were then selfed to produce an F2 population. Seventy-five percent of this F2 population was found to be kan^r, and thirty-three percent of the kan^r resistant plants showed the *ell* phenotype, confirming the recessive nature of this mutant. Consistent segregation of the *ell* phenotype and kan^r marker was also observed in a subsequent backcross, further indicating that *ell* was tagged by the T-DNA.

Genomic DNA blot analysis, using an NPTII probe derived from the T-DNA vector, showed that a unique single copy of T-DNA was integrated into the *ell* genome. This result, together with the segregation data described above, further indicated that the *ell* phenotype was associated with the kan^r marker, and that the *ell* mutation resulted from a single T-DNA insertion in the *Arabidopsis* genome.

The T-DNA-tagged locus was then isolated by constructing a genomic DNA library from the *ell* mutant and was mapped by hybridization using the NPTII probe. Fig. 10A shows the physical map of the T-DNA tagged locus that was determined by DNA hybridization. One of three genomic clones that were found to hybridize to the NPTII probe was partially sequenced and found to have a complete T-DNA insertion and flanking plant sequences. A segment of this genomic clone containing both T-DNA and plant sequences was then used to screen a genomic library that was prepared from wild-type plants. Two positive clones that were identified in this screen were then sequenced. The genomic nucleotide sequence is presented in Fig. 15 (SEQ ID NO: 3).

The T-DNA-plant DNA insert junctions were also used as probes to screen a cDNA library that was prepared from wild-type plants. One isolated cDNA clone, designated D13, was found to have a nucleotide sequence (SEQ ID NO: 1) that matched the genomic sequences flanking the right T-DNA border. Comparison of the cDNA (Fig. 14) with the genomic DNA sequence (Fig. 15) also revealed that the T-DNA was inserted at a location forty base pairs upstream of the 5' end of the *ELL* cDNA transcript (Fig. 16). The complete genomic fragment covering the cDNA sequence was composed of 14 exons and 13 introns (Fig. 10A). Probes that were prepared from both the cDNA or genomic clone were then used for DNA blot analysis. Results from this analysis confirmed that the *ELL* gene was of plant origin.

We also determined the chromosomal position of *ELL* by standard segregation analysis of restriction fragment length polymorphisms (RFLPs) in recombinant inbred lines (Nam et al., *Plant Cell* 1: 699, 1989; Lister and Dean, *Plant J.* 4: 745, 1993;

Hauge et al., *Plant J.* 3: 745, 1993; Schmidt et al., *Science* 270: 480, 1995; Zachgo et al., *Genomic Res.* 6: 19, 1996). By this analysis, we found that *ELL* is located on chromosome 3 and is flanked by the chromosomal markers by mi456 and g2778 (Fig. 10B).

ELL Encodes a Novel C-14 Sterol Reductase

A comparison of the deduced polypeptide sequence of the full-length *ELL* cDNA clone to the GenBank database showed that *ELL* had 35% identity to C-14 sterol reductase (Erg24) in yeast (Lorenz and Parks, *DNA Cell Biol.* 9: 685, 1992; Lai et al., *Gene* 140: 41, 1994) (Fig. 11) and 40% identity to the lamin B receptor (LBR) in humans (Ye and Worman, *J. Biol. Chem.* 269: 11306, 1994) (Fig. 12). In addition, the amino acid sequence of *ELL* predicted several hydrophobic regions and between eight to nine transmembrane domains, consistent with the yeast Erg24 and human LBR. However, *ELL* was observed to lack a basic nucleoplasmic amino-terminal domain of about 200 amino acids that has been identified in human LBR. Database searches also revealed that at least two *Arabidopsis* expression sequence tagged (EST) clones (GenBank accession numbers T45011 and T42407) shared homology to *ELL*. DNA sequencing revealed that T45011 encodes an unknown gene with 60% nucleotide sequence identity to *ELL*. The predicted amino acid sequence of T45011 was also observed to have greater than 50% identity to the yeast ERG24 and human LBR. These results further confirmed that *ELL* is encoded by a gene that is a member of the C-14 sterol reductase gene family. T42407 was found to encode an *Arabidopsis* sterol Δ7-reductase (Lecain et al., *J. Biol. Chem.* 271: 10866, 1996) that shares 32% amino acid identity to ELL.

RNA blot analysis indicated multiple transcripts hybridizing to the full-length *ELL* cDNA.

To determine whether the *ell* mutant phenotype is corrected by exogenous feeding of brassinolide, we germinated *ell* seedlings on agar plates containing 1 μM

brassinolide or 1 μM 24-epibrassinolide (Li et al., *Science* 272: 398, 1996). The results of these experiments showed that the presence of brassinolide or 24-epibrassinolide, in the growth medium of *ell* plants did not alter the mutant phenotype (Fig. 13A-13B). Thus, it appears that steroid compounds other than BRs are needed to restore an *ell* mutant to a normal growth and development phenotype, as reflected by the pleiotropic phenotypes such as stunted roots (Fig. 2) and impaired embryogenesis (Fig. 7A-7F).

To confirm that ELL activity was upstream of DET2 in the sterol biosynthesis pathway, a double mutant between *ell* and *det2* was constructed and analyzed. The phenotype of *det2/ell* was indistinguishable from *ell*, further supporting the hypothesis that DET2 was epistatic to ELL.

Isolation of Other C-14 Sterol Reductase cDNAs and Genomic DNAs

Based on the C-14 sterol reductase genes and polypeptides described herein, the isolation of additional plant C-14 sterol reductase coding sequences is made possible using standard strategies and techniques that are well known in the art. For example, using all or a portion of the amino acid sequence of a C-14 sterol reductase polypeptide, one may readily design C-14 sterol reductase-specific oligonucleotide probes, including C-14 sterol reductase degenerate oligonucleotide probes (i.e., a mixture of all possible coding sequences for a given amino acid sequence). These oligonucleotides may be based upon the sequence of either DNA strand and any appropriate portion of the C-14 sterol reductase sequence (for example, Fig. 14; SEQ ID NOS: 2 and 1, respectively; and Fig. 15 (SEQ ID NO: 3). General methods for designing and preparing such probes are provided, for example, in Ausubel et al., 1996, *Current Protocols in Molecular Biology*, Wiley Interscience, New York, and Berger and Kimmel, *Guide to Molecular Cloning Techniques*, 1987, Academic Press, New York. These oligonucleotides are useful for C-14 sterol reductase gene isolation, either through their use as probes capable of hybridizing to C-14 sterol reductase

complementary sequences or as primers for various amplification techniques, for example, polymerase chain reaction (PCR) cloning strategies.

Hybridization techniques and screening procedures are well known to those skilled in the art and are described, for example, in Ausubel et al. (*supra*); Berger and Kimmel (*supra*); and Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York. If desired, a combination of different oligonucleotide probes may be used for the screening of a recombinant DNA library. The oligonucleotides may be detectably-labeled using methods known in the art and used to probe filter replicas from a recombinant DNA library. Recombinant DNA libraries are prepared according to methods well known in the art, for example, as described in Ausubel et al. (*supra*), or they may be obtained from commercial sources.

For detection or isolation of closely related C-14 sterol reductase sequences having greater than 80% identity, high stringency conditions are preferably used; such conditions include hybridization at about 65°C and about 50% formamide, a first wash at about 65°C, about 2X SSC, and 1% SDS, followed by a second wash at about 65°C and about 0.1% SDS, and 0.1X SSC. Lower stringency conditions for detecting C-14 sterol reductase genes having about 40-50% sequence identity to the C-14 sterol reductase genes described herein include, for example, hybridization at about 37°C in the absence of formamide, a first wash at about 37°C, about 6X SSC, and about 1% SDS, and a second wash at about 37°C, about 6X SSC, and about 1% SDS. These stringency conditions are exemplary; other appropriate conditions may be determined by those skilled in the art.

As discussed above, C-14 sterol reductase oligonucleotides may also be used as primers in amplification cloning strategies, for example, using PCR. PCR methods are well known in the art and are described, for example, in *PCR Technology*, Erlich, ed., Stockton Press, London, 1989; *PCR Protocols: A Guide to Methods and Applications*, Innis et al., eds., Academic Press, Inc., New York, 1990; and Ausubel et

al. (*supra*). Primers are optionally designed to allow cloning of the amplified product into a suitable vector, for example, by including appropriate restriction sites at the 5' and 3' ends of the amplified fragment (as described herein). If desired, C-14 sterol reductase sequences may be isolated using the PCR "RACE" technique, or Rapid Amplification of cDNA Ends (see, e.g., Innis et al. (*supra*)). By this method, oligonucleotide primers based on a C-14 sterol reductase sequence are oriented in the 3' and 5' directions and are used to generate overlapping PCR fragments. These overlapping 3'- and 5'-end RACE products are combined to produce an intact full-length cDNA. This method is described in Innis et al. (*supra*); and Frohman et al., *Proc. Natl. Acad. Sci. USA* 85: 8998, 1988.

Alternatively, any plant cDNA expression library may be screened by functional complementation of a yeast C-14 reductase mutant (for example, the *erg24* mutant described by Lorenz and Parks, *DNA Cell Biol.* 9: 685, 1992) according to standard methods.

Useful C-14 sterol reductase sequences may be isolated from any appropriate organism. Confirmation of a sequence's relatedness to the C-14 sterol reductase polypeptide family may be accomplished by a variety of conventional methods including, but not limited to, functional complementation assays and sequence comparison. In addition, the activity of any C-14 sterol reductase sequence may be evaluated according to any of the techniques described herein.

C-14 Sterol Reductase Polypeptide Expression

C-14 sterol reductase polypeptides may be produced by transformation of a suitable host cell with all or part of a C-14 sterol reductase cDNA (for example, the cDNA described above) in a suitable expression vehicle or with a plasmid construct engineered for increasing the expression of a C-14 sterol reductase polypeptide (*supra*) *in vivo*.

Those skilled in the field of molecular biology will understand that any of a wide variety of expression systems may be used to provide the recombinant protein. The precise host cell used is not critical to the invention. The C-14 sterol reductase protein may be produced in a prokaryotic host, for example, *E. coli*, or in a eukaryotic host, for example, *Saccharomyces cerevisiae*, mammalian cells (for example, COS 1 or NIH 3T3 cells), or any of a number of plant cells including, without limitation, algae, tree species, ornamental species, temperate fruit species, tropical fruit species, vegetable species, legume species, monocots, dicots, or in any plant of commercial or agricultural significance. Particular examples of suitable plant hosts include, but are not limited to, Conifers, Petunia, Tomato, Potato, Tobacco, *Arabidopsis*, Lettuce, Sunflower, Oilseed rape, Flax, Cotton, Sugarbeet, Celery, Soybean, Alfalfa, *Medicago*, Lotus, *Vigna*, Cucumber, Carrot, Eggplant, Cauliflower, Horseradish, Morning Glory, Poplar, Walnut, Apple, Asparagus, Rice, Maize, Millet, Onion, Barley, Orchard grass, Oat, Rye, and Wheat.

Such cells are available from a wide range of sources including the American Type Culture Collection (Rockland, MD); or from any of a number seed companies, for example, W. Atlee Burpee Seed Co. (Warminster, PA), Park Seed Co. (Greenwood, SC), Johnny Seed Co. (Albion, ME), or Northrup King Seeds (Harstville, SC). Descriptions and sources of useful host cells are also found in Vasil I.K., *Cell Culture and Somatic Cell Genetics of Plants*, Vol I, II, III Laboratory Procedures and Their Applications Academic Press, New York, 1984; Dixon, R.A., *Plant Cell Culture-A Practical Approach*, IRL Press, Oxford University, 1985; Green et al., *Plant Tissue and Cell Culture*, Academic Press, New York, 1987; and Gasser and Fraley, *Science* 244: 1293, 1989.

For prokaryotic expression, DNA encoding a C-14 sterol reductase polypeptide is carried on a vector operably linked to control signals capable of effecting expression in the prokaryotic host. If desired, the coding sequence may contain, at its 5' end, a sequence encoding any of the known signal sequences capable of effecting secretion

of the expressed protein into the periplasmic space of the host cell, thereby facilitating recovery of the protein and subsequent purification. Prokaryotes most frequently used are various strains of *E. coli*; however, other microbial strains may also be used. Plasmid vectors are used which contain replication origins, selectable markers, and control sequences derived from a species compatible with the microbial host. Examples of such vectors are found in Pouwels et al. (*supra*) or Ausubel et al. (*supra*). Commonly used prokaryotic control sequences (also referred to as "regulatory elements") are defined herein to include promoters for transcription initiation, optionally with an operator, along with ribosome binding site sequences. Promoters commonly used to direct protein expression include the beta-lactamase (penicillinase), the lactose (lac) (Chang et al., *Nature* 198: 1056, 1977), the tryptophan (Trp) (Goeddel et al., *Nucl. Acids Res.* 8: 4057, 1980), and the tac promoter systems, as well as the lambda-derived P_L promoter and N-gene ribosome binding site (Simatake et al., *Nature* 292: 128, 1981).

One particular bacterial expression system for C-14 sterol reductase polypeptide production is the *E. coli* pET expression system (Novagen, Inc., Madison, WI). According to this expression system, DNA encoding a C-14 sterol reductase polypeptide is inserted into a pET vector in an orientation designed to allow expression. Since the C-14 sterol reductase gene is under the control of the T7 regulatory signals, expression of C-14 sterol reductase is induced by inducing the expression of T7 RNA polymerase in the host cell. This is typically achieved using host strains which express T7 RNA polymerase in response to IPTG induction. Once produced, recombinant C-14 sterol reductase polypeptide is then isolated according to standard methods known in the art, for example, those described herein.

Another bacterial expression system for C-14 sterol reductase polypeptide production is the pGEX expression system (Pharmacia). This system employs a GST gene fusion system which is designed for high-level expression of genes or gene fragments as fusion proteins with rapid purification and recovery of functional gene

products. The protein of interest is fused to the carboxyl terminus of the glutathione S-transferase protein from *Schistosoma japonicum* and is readily purified from bacterial lysates by affinity chromatography using Glutathione Sepharose 4B. Fusion proteins can be recovered under mild conditions by elution with glutathione. Cleavage of the glutathione S-transferase domain from the fusion protein is facilitated by the presence of recognition sites for site-specific proteases upstream of this domain. For example, proteins expressed in pGEX-2T plasmids may be cleaved with thrombin; those expressed in pGEX-3X may be cleaved with factor Xa.

For eukaryotic expression, the method of transformation or transfection and the choice of vehicle for expression of the C-14 sterol reductase polypeptide will depend on the host system selected. Transformation and transfection methods are described, e.g., in Ausubel et al. (*supra*); Weissbach and Weissbach, *Methods for Plant Molecular Biology*, Academic Press, 1989; Gelvin et al., *Plant Molecular Biology Manual*, Kluwer Academic Publishers, 1990; Kindle, K., *Proc. Natl. Acad. Sci., U.S.A* 87: 1228, 1990; Potrykus, I., *Annu. Rev. Plant Physiol. Plant Mol. Biology* 42: 205, 1991; and BioRad (Hercules, CA) Technical Bulletin #1687 (Biolistic Particle Delivery Systems). Expression vehicles may be chosen from those provided, e.g., in *Cloning Vectors: A Laboratory Manual* (P.H. Pouwels et al., 1985, Supp. 1987); Gasser and Fraley (*supra*); Clontech Molecular Biology Catalog (Catalog 1992/93 Tools for the Molecular Biologist, Palo Alto, CA); and the references cited above.

Most preferably, an C-14 sterol reductase polypeptide is produced by a stably-transfected plant cell line, a transiently-transfected plant cell line, or by a transgenic plant. A number of vectors suitable for stable transfection of plant cells or for the establishment of transgenic plants are available to the public; such vectors are described in Pouwels et al. (*supra*), Weissbach and Weissbach (*supra*), and Gelvin et al. (*supra*). Methods for constructing such cell lines are described in, e.g., Weissbach and Weissbach (*supra*), and Gelvin et al. (*supra*). Typically, plant expression vectors include (1) a cloned plant gene under the transcriptional control of 5' and 3' regulatory

sequences and (2) a dominant selectable marker. Such plant expression vectors may also contain, if desired, a promoter regulatory region (for example, one conferring inducible or constitutive, environmentally- or developmentally-regulated, or cell- or tissue-specific expression), a transcription initiation start site, a ribosome binding site, an RNA processing signal, a transcription termination site, and/or a polyadenylation signal.

Alternatively, the C-14 sterol reductase polypeptide may be produced using a transient expression system (e.g., the maize transient expression system described by Sheen, *Plant Cell* 2: 1027, 1990).

Once the desired C-14 sterol reductase nucleic acid sequences is obtained, it may be manipulated in a variety of ways known in the art. For example, where the sequence involves non-coding flanking regions, the flanking regions may be subjected to mutagenesis.

The C-14 sterol reductase DNA sequence of the invention may, if desired, be combined with other DNA sequences in a variety of ways. The C-14 sterol reductase DNA sequence of the invention may be employed with all or part of the gene sequences normally associated with the C-14 sterol reductase protein. In its component parts, a DNA sequence encoding a C-14 sterol reductase protein is combined in a DNA construct having a transcription initiation control region capable of promoting transcription and translation in a host cell.

In general, the constructs will involve regulatory regions functional in plants which provide for modified production of C-14 sterol reductase protein as discussed herein. The open reading frame coding for the C-14 sterol reductase protein or functional fragment thereof will be joined at its 5' end to a transcription initiation regulatory region such as the sequence naturally found in the 5' upstream region of the C-14 sterol reductase structural gene. Numerous other transcription initiation regions are available which provide for constitutive or inducible regulation.

For applications where developmental, cell, tissue, hormonal, or environmental expression is desired, appropriate 5' upstream non-coding regions are obtained from other genes, for example, from genes regulated during meristem development, seed development, embryo development, or leaf development.

Regulatory transcript termination regions may also be provided in DNA constructs of this invention as well. Transcript termination regions may be provided by the DNA sequence encoding the C-14 sterol reductase protein or any convenient transcription termination region derived from a different gene source. The transcript termination region will contain preferably at least 1-3 kb of sequence 3' to the structural gene from which the termination region is derived. Plant expression constructs having C-14 sterol reductase as the DNA sequence of interest for expression (in either the sense or antisense orientation) may be employed with a wide variety of plant life, particularly plant life involved in the production of storage reserves (for example, those involving carbon and nitrogen metabolism). Such genetically-engineered plants are useful for a variety of industrial and agricultural applications as discussed below. Importantly, this invention is applicable to dicotyledons and monocotyledons, and will be readily applicable to any new or improved transformation or regeneration method.

An example of a useful plant promoter according to the invention is a caulimovirus promoter, for example, a cauliflower mosaic virus (CaMV) promoter. These promoters confer high levels of expression in most plant tissues, and the activity of these promoters is not dependent on virally encoded proteins. CaMV is a source for both the 35S and 19S promoters. In most tissues of transgenic plants, the CaMV 35S promoter is a strong promoter (see, e.g., Odell et al., *Nature* 313: 810 1985). The CaMV promoter is also highly active in monocots (see, e.g., Dekeyser et al., *Plant Cell* 2: 591, 1990; Terada and Shimamoto, *Mol. Gen. Genet.* 220: 389, 1990). Moreover, activity of this promoter can be further increased (i.e., between 2-10 fold) by duplication of the CaMV 35S promoter (see e.g., Kay et al., *Science* 236: 1299,

1987; Ow et al., *Proc. Natl. Acad. Sci., U.S.A.* 84: 4870, 1987; and Fang et al., *Plant Cell* 1: 141, 1989).

Other useful plant promoters include, without limitation, the nopaline synthase promoter (An et al., *Plant Physiol.* 88: 547, 1988) and the octopine synthase promoter (Fromm et al., *Plant Cell* 1: 977, 1989).

For certain applications, it may be desirable to produce the C-14 sterol reductase gene product in an appropriate tissue, at an appropriate level, or at an appropriate developmental time. For this purpose, there are an assortment of gene promoters, each with its own distinct characteristics embodied in its regulatory sequences, shown to be regulated in response to the environment, hormones, and/or developmental cues. These include gene promoters that are responsible for heat-regulated gene expression (see, e.g., Callis et al., *Plant Physiol.* 88: 965, 1988; Takahashi and Komeda, *Mol. Gen. Genet.* 219: 365, 1989; and Takahashi et al. *Plant J.* 2: 751, 1992), light-regulated gene expression (e.g., the pea *rbcS-3A* described by Kuhlemeier et al., *Plant Cell* 1: 471, 1989; the maize *rbcS* promoter described by Schäffner and Sheen, *Plant Cell* 3: 997, 1991; or the cholorphyll a/b-binding protein gene found in pea described by Simpson et al., *EMBO J.* 4: 2723, 1985), hormone-regulated gene expression (for example, the abscisic acid (ABA) responsive sequences from the *Em* gene of wheat described by Marcotte et al., *Plant Cell* 1: 969, 1989; the ABA-inducible HVA1 and HVA22, and rd29A promoters described for barley and *Arabidopsis* by Straub et al., *Plant Cell* 6: 617, 1994, Shen et al., *Plant Cell* 7: 295, 1995; and wound-induced gene expression (for example, of *wunI* described by Siebertz et al., *Plant Cell* 1: 961, 1989), or organ-specific gene expression (for example, of the tuber-specific storage protein gene described by Roshal et al., *EMBO J.* 6: 1155, 1987; the 23-kDa zein gene from maize described by Schernthaner et al., *EMBO J.* 7: 1249, 1988; or the French bean ß-phaseolin gene described by Bustos et al., *Plant Cell* 1: 839, 1989).

Plant expression vectors may also optionally include RNA processing signals, e.g, introns, which have been shown to be important for efficient RNA synthesis and accumulation (Callis et al., *Genes and Dev.* 1: 1183, 1987). The location of the RNA splice sequences can dramatically influence the level of transgene expression in plants. In view of this fact, an intron may be positioned upstream or downstream of a C-14 sterol reductase polypeptide-encoding sequence in the transgene to modulate levels of gene expression.

In addition to the aforementioned 5' regulatory control sequences, the expression vectors may also include regulatory control regions which are generally present in the 3' regions of plant genes (Thornburg et al., *Proc. Natl. Acad. Sci. U.S.A.* 84: 744, 1987; An et al., *Plant Cell* 1: 115, 1989). For example, the 3' terminator region may be included in the expression vector to increase stability of the mRNA. One such terminator region may be derived from the PI-II terminator region of potato. In addition, other commonly used terminators are derived from the octopine or nopaline synthase signals.

The plant expression vector also typically contains a dominant selectable marker gene used to identify those cells that have become transformed. Useful selectable genes for plant systems include genes encoding antibiotic resistance genes, for example, those encoding resistance to hygromycin, kanamycin, bleomycin, G418, streptomycin, or spectinomycin. Genes required for photosynthesis may also be used as selectable markers in photosynthetic-deficient strains. Alternatively, the green-fluorescent protein from the jellyfish *Aequorea victoria* may be used as a selectable marker (Sheen et al., *Plant J.* 8:777, 1995; Chiu et al., *Current Biology* 6: 325, 1996). Finally, genes encoding herbicide resistance may be used as selectable markers; useful herbicide resistance genes include the *bar* gene encoding the enzyme phosphinothricin acetyltransferase and conferring resistance to the broad spectrum herbicide Basta® (Hoechst AG, Frankfurt, Germany).

Efficient use of selectable markers is facilitated by a determination of the susceptibility of a plant cell to a particular selectable agent and a determination of the concentration of this agent which effectively kills most, if not all, of the transformed cells. Some useful concentrations of antibiotics for tobacco transformation include, e.g., 75-100 μg/ml (kanamycin), 20-50 μg/ml (hygromycin), or 5-10 μg/ml (bleomycin). A useful strategy for selection of transformants for herbicide resistance is described, e.g., by Vasil et al., *supra*.

It should be readily apparent to one skilled in the art of molecular biology, especially in the field of plant molecular biology, that the level of gene expression is dependent, not only on the combination of promoters, RNA processing signals, and terminator elements, but also on how these elements are used to increase the levels of selectable marker gene expression.

Plant Transformation

Upon construction of the plant expression vector, several standard methods are available for introduction of the vector into a plant host, thereby generating a transgenic plant. These methods include (1) Agrobacterium-mediated transformation (*A. tumefaciens* or *A. rhizogenes*) (see, e.g., Lichtenstein and Fuller In: *Genetic Engineering*, vol 6, PWJ Rigby, ed, London, Academic Press, 1987; and Lichtenstein, C.P., and Draper, J,. In: *DNA Cloning*, Vol II, D.M. Glover, ed, Oxford, IRI Press, 1985)), (2) the particle delivery system (see, e.g., Gordon-Kamm et al., *Plant Cell* 2: 603 (1990); or BioRad Technical Bulletin 1687, *supra*), (3) microinjection protocols (see, e.g., Green et al., *supra*), (4) polyethylene glycol (PEG) procedures (see, e.g., Draper et al., *Plant Cell Physiol.* 23: 451, 1982; or e.g., Zhang and Wu, *Theor. Appl. Genet.* 76: 835, 1988), (5) liposome-mediated DNA uptake (see, e.g., Freeman et al., *Plant Cell Physiol.* 25: 1353, 1984), (6) electroporation protocols (see, e.g., Gelvin et al., *supra*; Dekeyser et al., *supra*; Fromm et al., *Nature* 319: 791, 1986; Sheen *Plant Cell* 2: 1027, 1990; or Jang and Sheen *Plant Cell* 6: 1665, 1994), and (7) the vortexing

method (see, e.g., Kindle *supra*). The method of transformation is not critical to the invention. Any method which provides for efficient transformation may be employed. As newer methods are available to transform crops or other host cells, they may be directly applied.

The following is an example outlining one particular technique, an *Agrobacterium*-mediated plant transformation. By this technique, the general process for manipulating genes to be transferred into the genome of plant cells is carried out in two phases. First, cloning and DNA modification steps are carried out in *E. coli*, and the plasmid containing the gene construct of interest is transferred by conjugation or electroporation into *Agrobacterium*. Second, the resulting *Agrobacterium* strain is used to transform plant cells. Thus, for the generalized plant expression vector, the plasmid contains an origin of replication that allows it to replicate in *Agrobacterium* and a high copy number origin of replication functional in *E. coli*. This permits facile production and testing of transgenes in *E. coli* prior to transfer to *Agrobacterium* for subsequent introduction into plants. Resistance genes can be carried on the vector, one for selection in bacteria, for example, streptomycin, and another that will function in plants, for example, a gene encoding kanamycin resistance or herbicide resistance. Also present on the vector are restriction endonuclease sites for the addition of one or more transgenes and directional T-DNA border sequences which, when recognized by the transfer functions of *Agrobacterium*, delimit the DNA region that will be transferred to the plant.

In another example, plant cells may be transformed by shooting into the cell tungsten microprojectiles on which cloned DNA is precipitated. In the Biolistic Apparatus (Bio-Rad) used for the shooting, a gunpowder charge (22 caliber Power Piston Tool Charge) or an air-driven blast drives a plastic macroprojectile through a gun barrel. An aliquot of a suspension of tungsten particles on which DNA has been precipitated is placed on the front of the plastic macroprojectile. The latter is fired at an acrylic stopping plate that has a hole through it that is too small for the

macroprojectile to pass through. As a result, the plastic macroprojectile smashes against the stopping plate, and the tungsten microprojectiles continue toward their target through the hole in the plate. For the instant invention the target can be any plant cell, tissue, seed, or embryo. The DNA introduced into the cell on the microprojectiles becomes integrated into either the nucleus or the chloroplast.

In general, transfer and expression of transgenes in plant cells are now routine practices to those skilled in the art, and have become major tools to carry out gene expression studies in plants and to produce improved plant varieties of agricultural or commercial interest.

Transgenic Plant Regeneration

Plant cells transformed with a plant expression vector can be regenerated, for example, from single cells, callus tissue, or leaf discs according to standard plant tissue culture techniques. It is well known in the art that various cells, tissues, and organs from almost any plant can be successfully cultured to regenerate an entire plant; such techniques are described, e.g., in Vasil *supra*; Green et al., *supra*; Weissbach and Weissbach, *supra*; and Gelvin et al., *supra*.

In one particular example, a cloned C-14 sterol reductase polypeptide or an antisense construct under the control of the 35S CaMV promoter and the nopaline synthase terminator and carrying a selectable marker (for example, kanamycin resistance) is transformed into *Agrobacterium*. Transformation of leaf discs (for example, of tobacco leaf discs), with vector-containing *Agrobacterium* is carried out as described by Horsch et al. (*Science* 227: 1229, 1985). Putative transformants are selected after a few weeks (for example, 3 to 5 weeks) on plant tissue culture media containing kanamycin (e.g. 100 µg/ml). Kanamycin-resistant shoots are then placed on plant tissue culture media without hormones for root initiation. Kanamycin-resistant plants are then selected for greenhouse growth. If desired, seeds from self-fertilized transgenic plants can then be sowed in a soil-less medium and grown in a

greenhouse. Kanamycin-resistant progeny are selected by sowing surfaced sterilized seeds on hormone-free kanamycin-containing media. Analysis for the integration of the transgene is accomplished by standard techniques (see, for example, Ausubel et al. *supra*; Gelvin et al. *supra*).

Transgenic plants expressing the selectable marker are then screened for transmission of the transgene DNA by standard immunoblot and DNA detection techniques. Each positive transgenic plant and its transgenic progeny are unique in comparison to other transgenic plants established with the same transgene. Integration of the transgene DNA into the plant genomic DNA is in most cases random, and the site of integration can profoundly affect the levels and the tissue and developmental patterns of transgene expression. Consequently, a number of transgenic lines are usually screened for each transgene to identify and select plants with the most appropriate expression profiles.

Transgenic lines are evaluated for levels of transgene expression. Expression at the RNA level is determined initially to identify and quantitate expression-positive plants. Standard techniques for RNA analysis are employed and include PCR amplification assays using oligonucleotide primers designed to amplify only transgene RNA templates and solution hybridization assays using transgene-specific probes (see, e.g., Ausubel et al., *supra*). The RNA-positive plants are then analyzed for protein expression by Western immunoblot analysis using C-14 sterol reductase specific antibodies (see, e.g., Ausubel et al., *supra*). In addition, *in situ* hybridization and immunocytochemistry according to standard protocols can be done using transgene-specific nucleotide probes and antibodies, respectively, to localize sites of expression within transgenic tissue.

Once the recombinant C-14 sterol reductase protein is expressed in any cell or in a transgenic plant (for example, as described above), it may be isolated, e.g., using affinity chromatography. In one example, an anti-C14 sterol reductase antibody (e.g., produced as described in Ausubel et al., *supra*, or by any standard technique) may be

attached to a column and used to isolate the polypeptide. Lysis and fractionation of C-14 sterol reductase-producing cells prior to affinity chromatography may be performed by standard methods (see, e.g., Ausubel et al., *supra*). Once isolated, the recombinant protein can, if desired, be further purified, for example, by high performance liquid chromatography (see, e.g., Fisher, *Laboratory Techniques In Biochemistry And Molecular Biology*, eds., Work and Burdon, Elsevier, 1980).

Polypeptides of the invention, particularly short C-14 sterol reductase protein fragments, can also be produced by chemical synthesis (e.g., by the methods described in *Solid Phase Peptide Synthesis*, 2nd ed., 1984 The Pierce Chemical Co., Rockford, IL).

These general techniques of polypeptide expression and purification can also be used to produce and isolate useful C-14 sterol reductase fragments or analogs.

Antibodies

C-14 sterol reductases described herein (or immunogenic fragments or analogs) may be used to raise antibodies useful in the invention; such polypeptides may be produced by recombinant or peptide synthetic techniques (see, e.g., *Solid Phase Peptide Synthesis*, 2nd ed., 1984, Pierce Chemical Co., Rockford, IL; Ausubel et al., *supra*). The peptides may be coupled to a carrier protein, such as KLH as described in Ausubel et al, *supra*. The KLH-peptide is mixed with Freund's adjuvant and injected into guinea pigs, rats, or preferably rabbits. Antibodies may be purified by peptide antigen affinity chromatography.

Monoclonal antibodies may be prepared using the C-14 sterol reductase polypeptides described above and standard hybridoma technology (see, e.g., Kohler et al., *Nature* 256:495, 1975; Kohler et al., *Eur. J. Immunol.* 6:511, 1976; Kohler et al., *Eur. J. Immunol.* 6:292, 1976; Hammerling et al., *In Monoclonal Antibodies and T Cell Hybridomas*, Elsevier, NY, 1981; Ausubel et al., *supra*).

Once produced, polyclonal or monoclonal antibodies are tested for specific C-14 sterol reductase recognition by Western blot or immunoprecipitation analysis (by the methods described in Ausubel et al., *supra*). Antibodies which specifically recognize C-14 sterol reductases are considered to be useful in the invention; such antibodies may be used, e.g., in an immunoassay to monitor the level of C-14 sterol reductase produced by a plant.

Use

Because the present invention provides for the genetic manipulation of a plant sterol biosynthetic pathway, this invention described is useful for a variety of agricultural and commercial purposes including, but not limited to, increasing crop yields, improving crop and ornamental quality, and reducing agricultural production costs. For example, the methods, DNA constructs, proteins, and transgenic plants described herein are useful for improving a number of fruit and vegetable characteristics including, but not limited to, texture, size, nutritional content, modification of sterol composition, disease and insect resistance, and ripening processes. In addition, genetic manipulation of plant sterol composition (for example, seed sterol composition) is useful for improving food quality and oil stability, and regulating the formation of compounds having anti-nutritional properties.

In one particular example, antisense C-14 sterol reductase sequences are useful for reducing the expression of C-14 sterol reductase expression in a transgenic plant. Such reduced expression of C-14 sterol reductase provides a means for increasing the life-span of such plants. Increased life-span extends reproductive period, delays senescence, and increases branch number for high productivity and yield. In addition, transgenic plants expressing antisense C-14 sterol reductase are useful for producing plants having reduced and more compact proportions. Such plants require less space and land requirements for their growth, and are more convenient and efficient to harvest.

Overproduction of the C-14 sterol reductase in transgenic plants is useful for enhancing the production of steroid compounds having a variety of medicinal or agricultural applications. For example, overproduction of mammalian steroid hormones in plants offers an inexpensive means for producing such hormones.

In addition, C-14 sterol reductase polypeptides disclosed herein are useful for the development of enzyme inhibitors of the sterol biosynthetic pathway.

Other Embodiments

In other embodiments, the invention includes any protein which is substantially identical to a crucifer C-14 sterol reductase polypeptide (Fig. 10; SEQ ID NO:1); such homologs include other substantially pure naturally-occurring plant C-14 sterol reductase proteins as well as allelic variants; natural mutants; induced mutants; proteins encoded by DNA that hybridizes to the C-14 sterol reductase DNA sequence of Fig. 14 (SEQ ID NO: 2) under high stringency conditions or, less preferably, under low stringency conditions (e.g., washing at 2X SSC at 37°C with a probe length of at least 10-15 nucleotides), both as described herein; and proteins specifically bound by antisera directed to a C-14 sterol reductase polypeptide. The term also includes chimeric polypeptides that include a C-14 sterol reductase portion.

The invention further includes analogs of any naturally-occurring plant C-14 sterol reductase polypeptide. Analogs can differ from the naturally-occurring C-14 sterol reductase protein by amino acid sequence differences, by post-translational modifications, or by both. Analogs of the invention will generally exhibit at least 30%, more preferably 40%, and most preferably 50% or even 80-95% identity with all or part of a naturally-occurring plant C-14 sterol reductase amino acid sequence. The length of sequence comparison is at least 15 amino acid residues, preferably at least 25 amino acid residues, and more preferably more than 35 amino acid residues. Modifications include *in vivo* and *in vitro* chemical derivatization of polypeptides, e.g., acetylation, carboxylation, phosphorylation, or glycosylation; such modifications

may occur during polypeptide synthesis or processing or following treatment with isolated modifying enzymes. Analogs can also differ from the naturally-occurring C-14 sterol reductase polypeptide by alterations in primary sequence. These include genetic variants, both natural and induced (for example, resulting from random mutagenesis by irradiation or exposure to ethanemethylsulfate or by site-specific mutagenesis as described in Sambrook, Fritsch and Maniatis, *Molecular Cloning: A Laboratory Manual* (2d ed.), CSH Press, 1989, or Ausubel et al., *supra*). Also included are cyclized peptides, molecules, and analogs which contain residues other than L-amino acids, e.g., D-amino acids or non-naturally occurring or synthetic amino acids, e.g., β or γ amino acids.

In addition to full-length polypeptides, the invention also includes C-14 sterol reductase polypeptide fragments. As used herein, the term "fragment," means at least 20 contiguous amino acids, preferably at least 30 contiguous amino acids, more preferably at least 50 contiguous amino acids, and most preferably at least 60 to 80 or more contiguous amino acids. Fragments of C-14 sterol reductase polypeptides can be generated by methods known to those skilled in the art or may result from normal protein processing (e.g., removal of amino acids from the nascent polypeptide that are not required for biological activity or removal of amino acids by alternative mRNA splicing or alternative protein processing events).

Furthermore, the invention includes nucleotide sequences that facilitate specific detection of a C-14 sterol reductase nucleic acid. Thus, C-14 sterol reductase sequences described herein (e.g., SEQ ID NO: 2 and 3) or portions thereof may be used as probes to hybridize to nucleotide sequences from other plants (e.g., dicots, monocots, gymnosperms, and algae) by standard hybridization techniques under conventional conditions. Sequences that hybridize to a C-14 sterol reductase coding sequence or its complement and that encode a C-14 sterol reductase are considered useful in the invention. As used herein, the term "fragment," as applied to nucleic acid sequences, means at least 5 contiguous nucleotides, preferably at least 10 contiguous

nucleotides, more preferably at least 20 to 30 contiguous nucleotides, and most preferably at least 40 to 80 or more contiguous nucleotides. Fragments of C-14 sterol reductase nucleic acid sequences can be generated by methods known to those skilled in the art.

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference.

Other embodiments are within the following claims.

What is claimed is:

Claims

1. A substantially pure plant C-14 sterol reductase polypeptide.

2. A substantially pure polypeptide comprising an amino acid sequence substantially identical to the sequence shown in Fig. 14 (SEQ ID NO: 1).

3. The polypeptide of claim 1 or 2, wherein said polypeptide comprises the amino acid sequence shown in Fig. 14 (SEQ ID NO: 1).

4. The polypeptide of claim 1 or 2, wherein said polypeptide is from a dicot.

5. The polypeptide of claim 4, wherein said dicot is a crucifer.

6. A purified DNA encoding a C-14 sterol reductase polypeptide.

7. A purified DNA comprising a sequence substantially identical to the DNA sequence shown in Fig. 14 (SEQ ID NO: 2).

8. The purified DNA of claim 6 or 7, wherein said DNA comprises the sequence shown in Fig. 14 (SEQ ID NO: 2).

9. The purified DNA of claim 6 or 7, wherein said DNA encodes a polypeptide which has an amino acid sequence substantially identical to that shown in Fig. 14 (SEQ ID NO: 1).

10. The purified DNA of claim 9, wherein said DNA encodes a polypeptide which has the amino acid sequence shown in Fig. 14 (SEQ ID NO: 1).

11. The purified DNA of claim 6 or 7, wherein said DNA is from a dicot.

12. The purified DNA of claim 11, wherein said dicot is a crucifer.

13. A vector comprising the purified DNA of claim 6 or 7.

14. A cell comprising the purified DNA of claim 6 or 7.

15. A method of producing a recombinant C-14 sterol reductase polypeptide comprising

providing a cell transformed with purified DNA encoding a C-14 sterol reductase polypeptide positioned for expression in said cell, and

culturing said transformed cell under conditions for expressing said DNA.

16. The method of claim 15, wherein said method further comprises recovering said recombinant C-14 sterol reductase polypeptide.

17. The method of claim 15, wherein said cell is a plant cell.

18. A recombinant C-14 sterol reductase produced by the method of claim 15.

19. An isolated antibody which specifically recognizes and binds a plant C-14 sterol reductase polypeptide.

20. A transgenic plant which contains DNA encoding a C-14 sterol reductase polypeptide integrated into the genome of said plant, wherein said DNA is expressed in said transgenic plant.

21. A transgenic plant which contains DNA encoding an amino acid sequence substantially identical to the sequence shown in Fig. 14 (SEQ ID NO: 1) integrated into the genome of said plant, wherein said DNA is expressed in said transgenic plant.

22. A seed from a transgenic plant of claim 20 or 21.

23. A cell from a transgenic plant of claim 20 or 21.

24. A method of detecting a C-14 sterol reductase gene in a plant cell comprising:

contacting the purified DNA of claim 6 or 7 or a portion thereof greater than about 12 nucleotides in length with a preparation of genomic DNA from said plant cell under hybridization conditions providing detection of DNA sequences having about 40% or greater sequence identity to SEQ ID NO: 2.

25. A method of isolating a C-14 sterol reductase gene or portion thereof, said method comprising

(a) amplifying said C-14 sterol reductase gene using oligonucleotide primers, wherein said primers each have regions of complementarity to opposite DNA strands in a region of SEQ ID NO: 2; and

(b) isolating said C-14 sterol reductase gene or portion thereof.

26. A method for reducing the level of a C-14 sterol reductase polypeptide in a transgenic plant cell, said method comprising expressing in a plant cell an antisense C-14 sterol reductase nucleic acid sequence.

27. The method of claim 26, wherein said antisense C-14 sterol reductase nucleic acid sequence is encoded by a transgene integrated into the genome of said transgenic plant cell.

28. The method of claim 26, wherein said C-14 sterol reductase sequence is SEQ ID NO: 2 or SEQ ID NO: 3.

29. The method of claim 26, wherein said method further comprises growing a transgenic plant from said transgenic plant cell, whereby the level of the C-14 sterol reductase polypeptide is reduced in said transgenic plant.

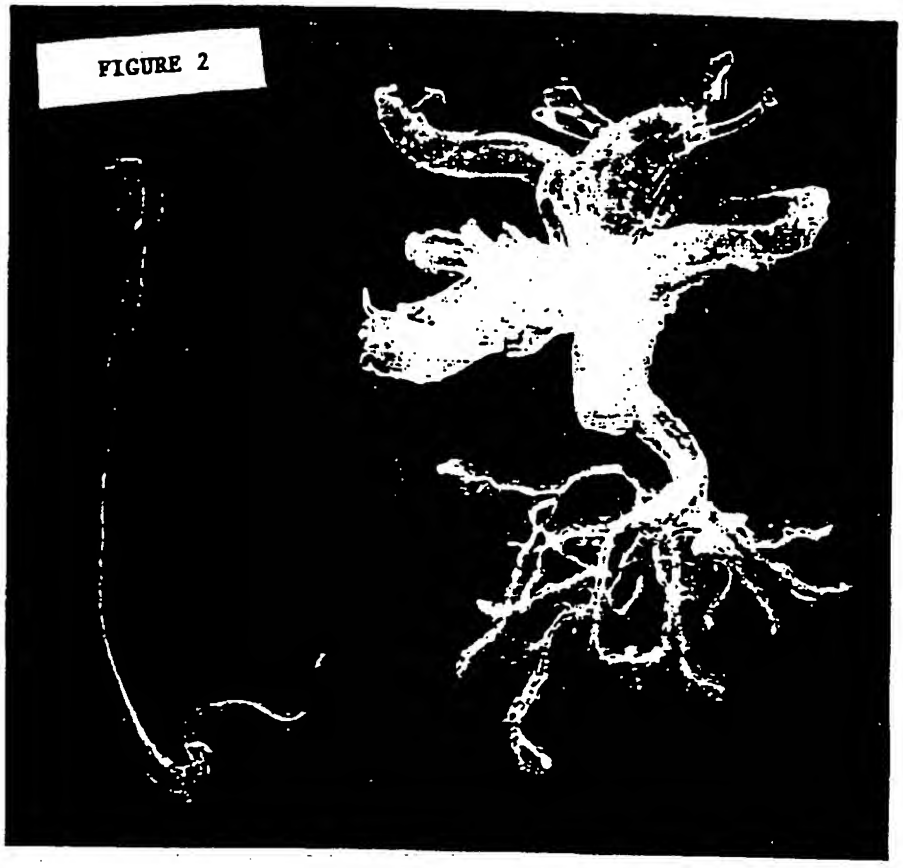
30. A method for increasing the level of a C-14 sterol reductase in a transgenic plant cell, comprising expressing in said transgenic plant cell a nucleic acid sequence encoding a polypeptide substantially identical to SEQ ID NO: 1.

31. A transgenic plant comprising purified DNA encoding a plant C-14 sterol reductase polypeptide, said DNA comprising a knockout mutation in said C-14 sterol reductase sequence.

FIGURE 1A

FIGURE 1B

FIGURE 2



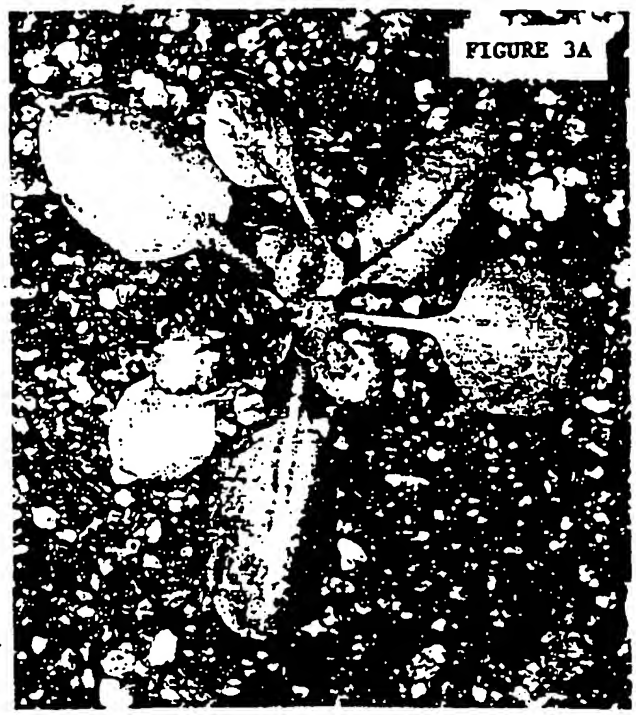


FIGURE 3A

FIGURE 3B

FIGURE 4

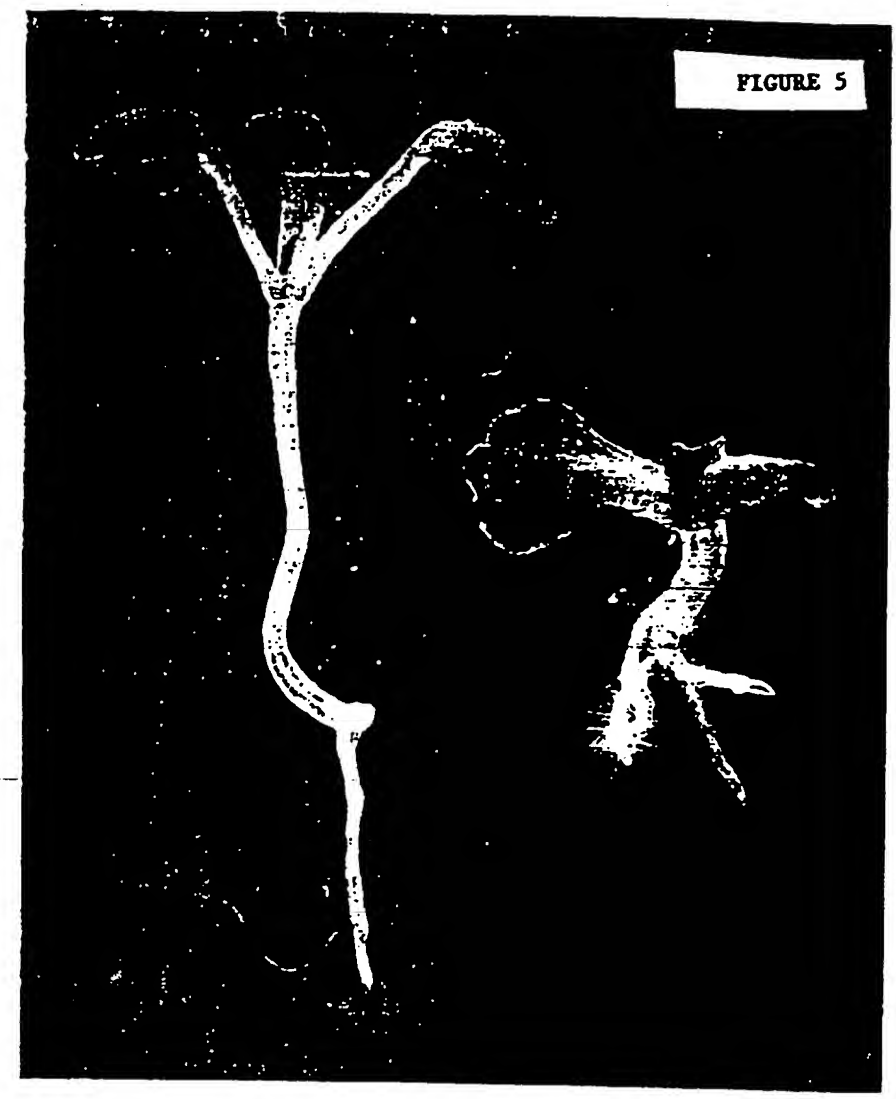


FIGURE 5

FIGURE 6A

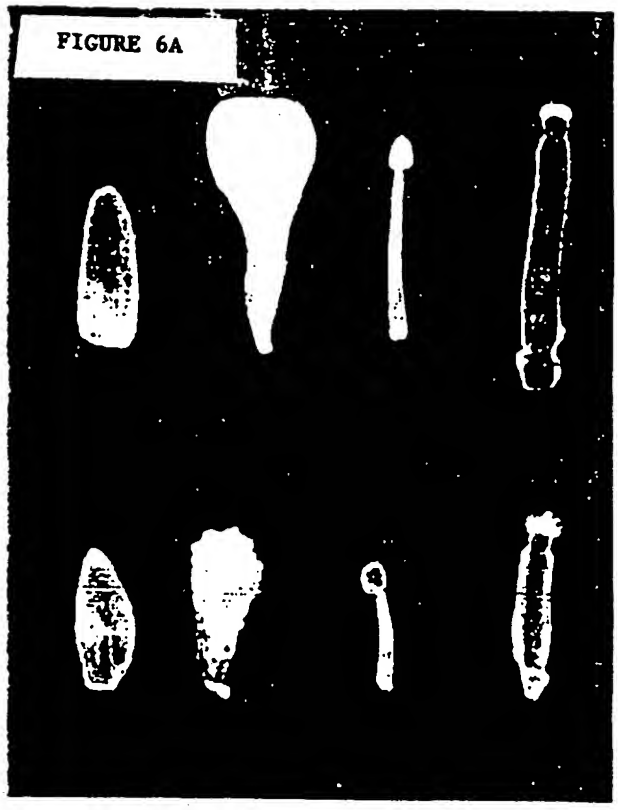


FIGURE 6B

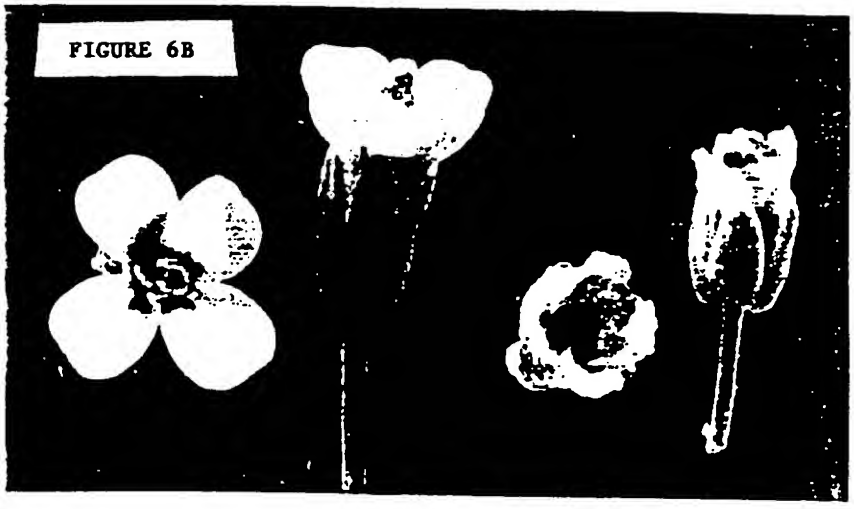


FIGURE 7A

FIGURE 7D

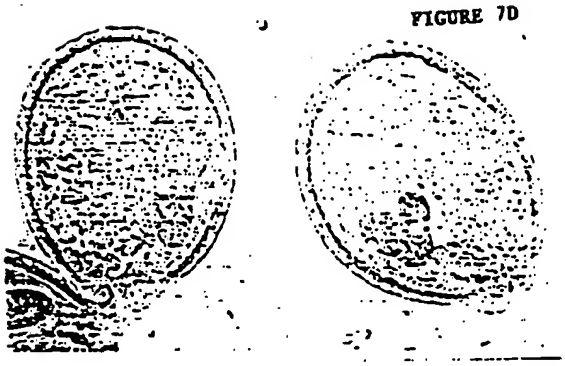


FIGURE 7B

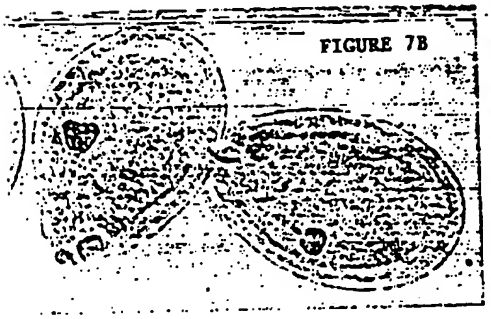


FIGURE 7E

FIGURE 7C

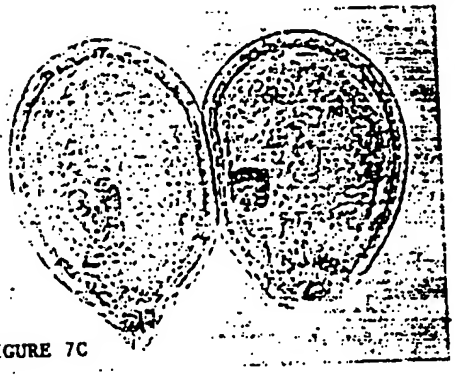


FIGURE 7F

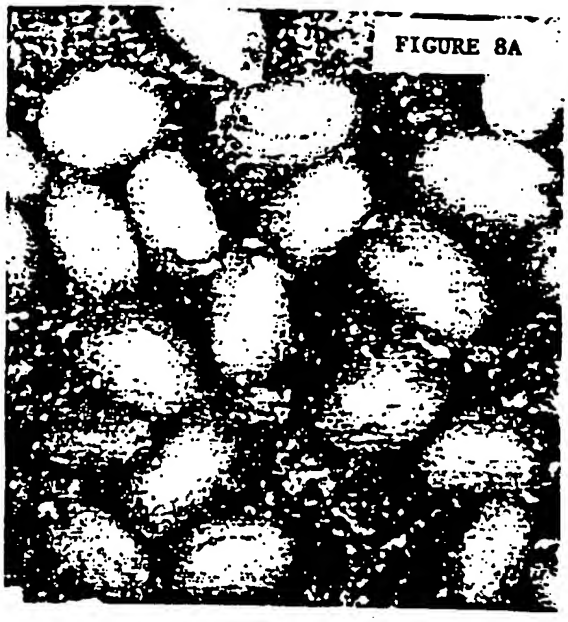


FIGURE 8A

FIGURE 8B

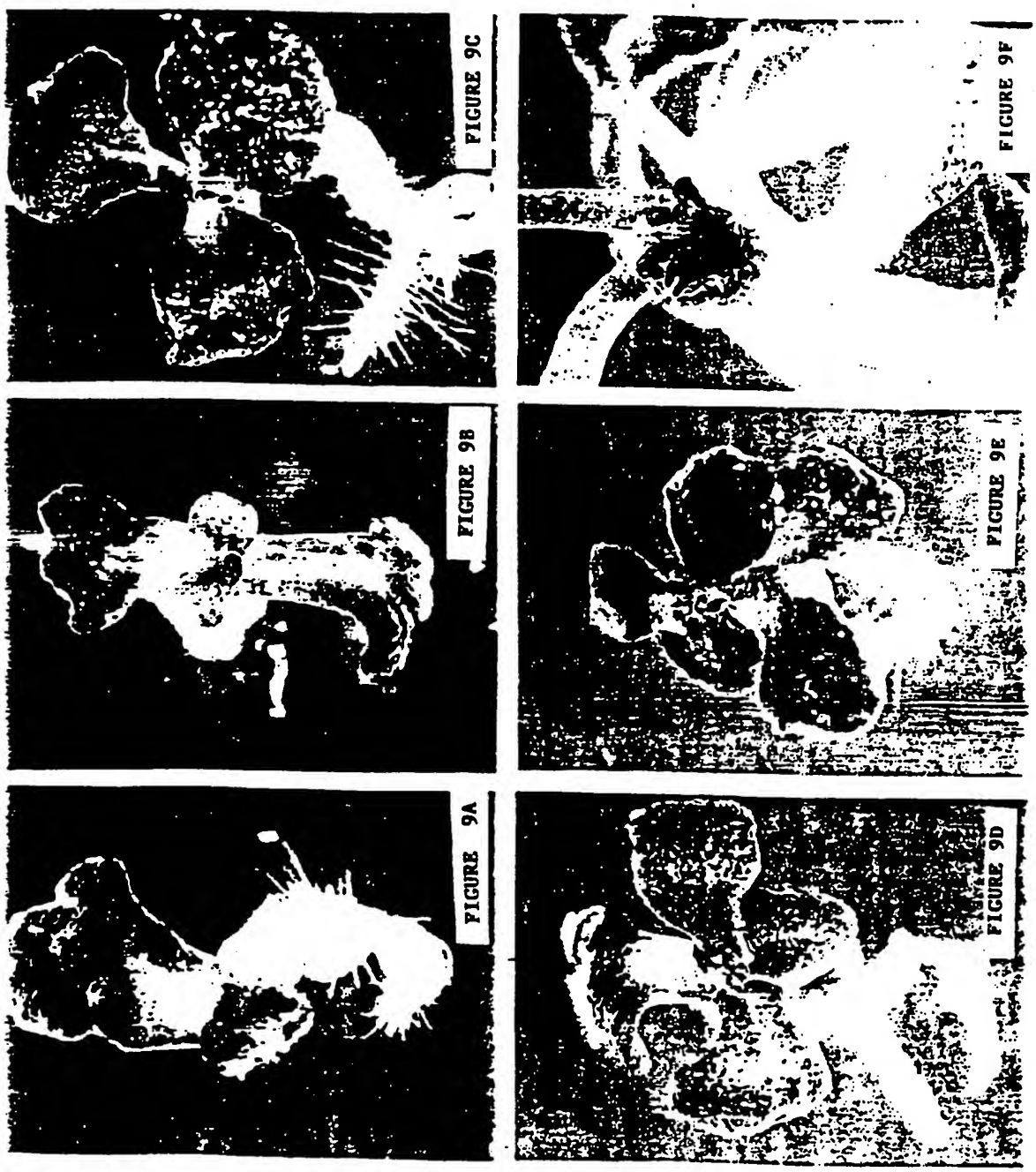


FIGURE 9A

FIGURE 9B

FIGURE 9C

FIGURE 9D

FIGURE 9E

FIGURE 9F

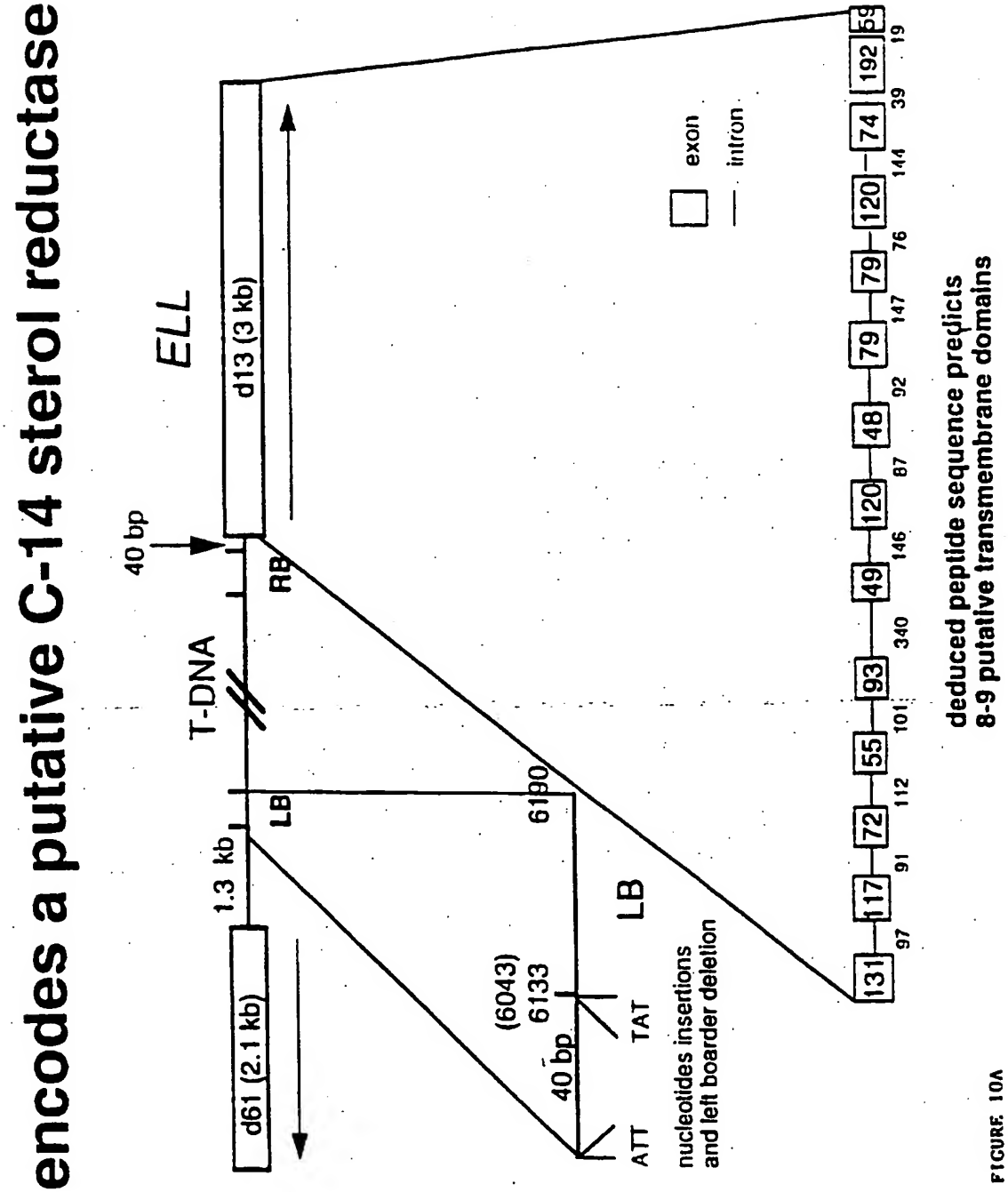

ELL encodes a putative C-14 sterol reductase
ELL
d13 (3 kb)
d61 (2.1 kb)
40 bp
RB
T-DNA
LB
1.3 kb
6190
(6043)
6133
40 bp
ATT
TAT
LB
nucleotides insertions
and left boarder deletion
exon
intron
131
117
72
55
93
49
120
48
79
79
120
74
192
59
97
91
112
101
340
146
87
92
147
76
144
39
19
deduced peptide sequence predicts
8-9 putative transmembrane domains

FIGURE 10A

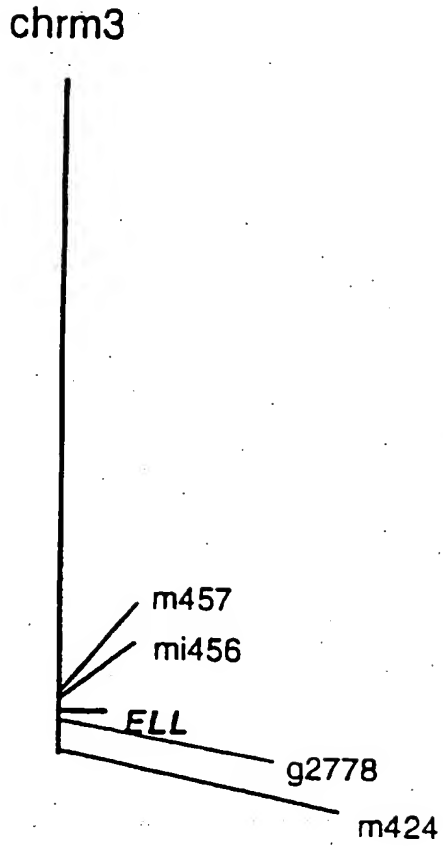


FIGURE 10B

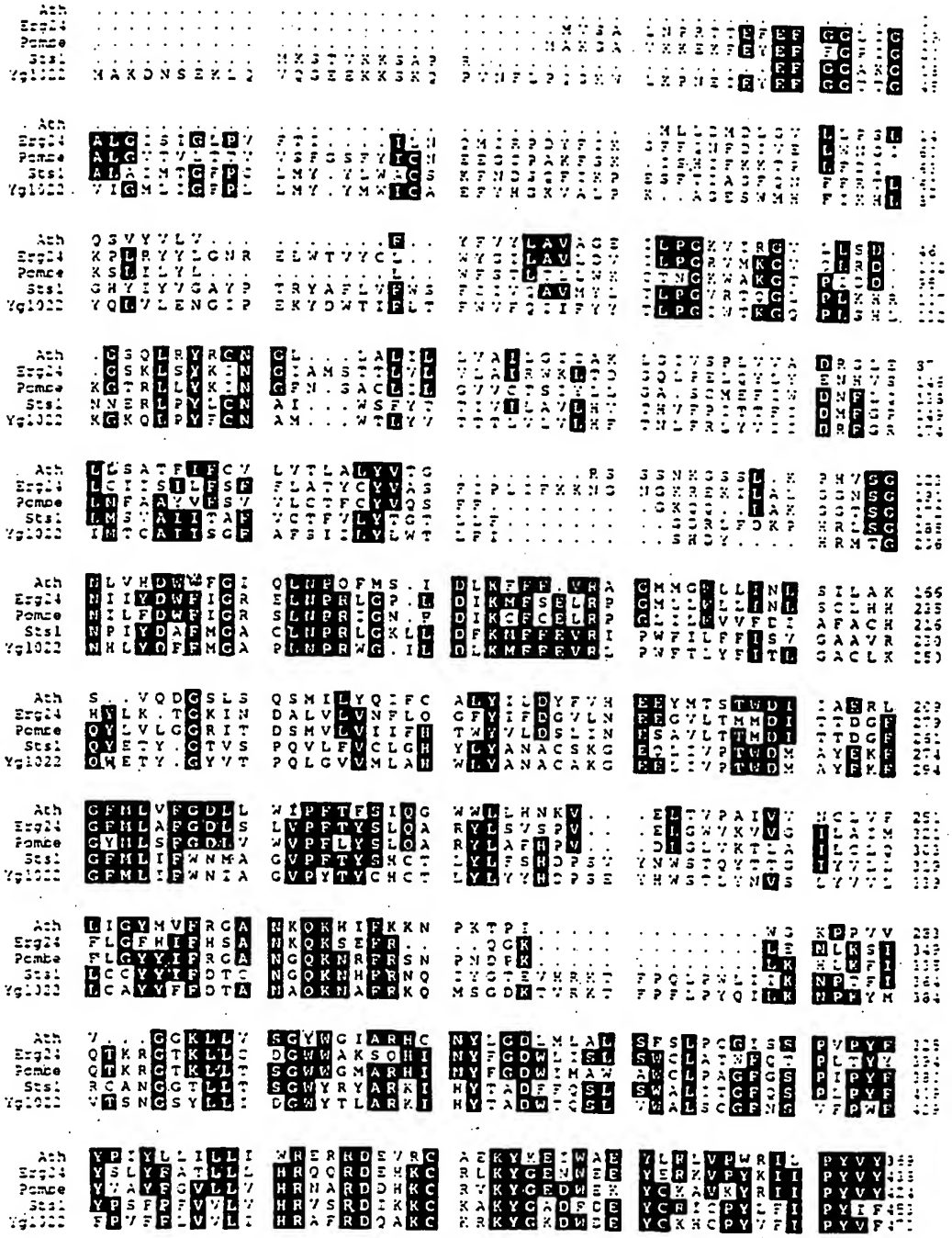


FIGURE 11

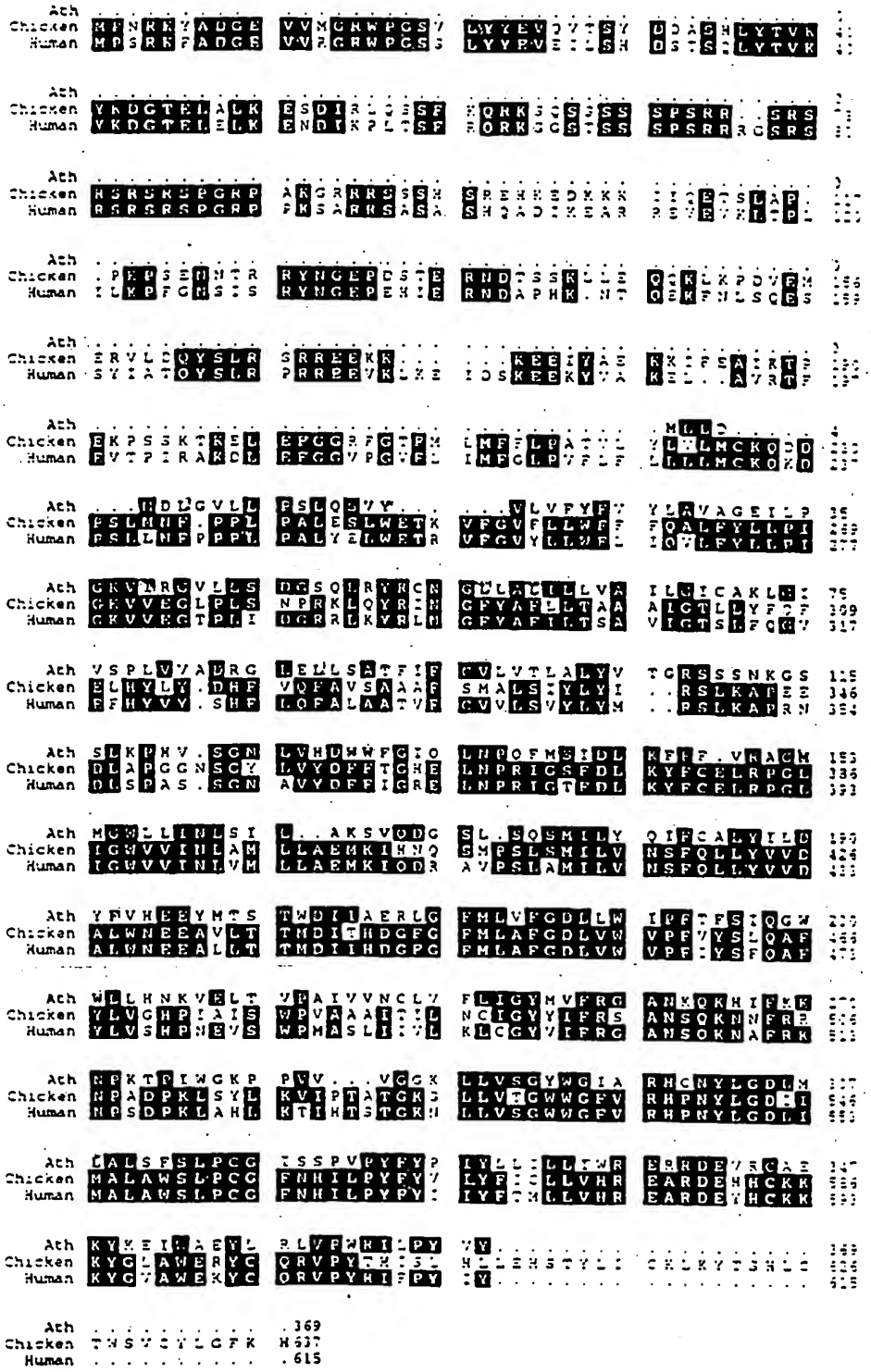


FIGURE 12

FIGURE 13A

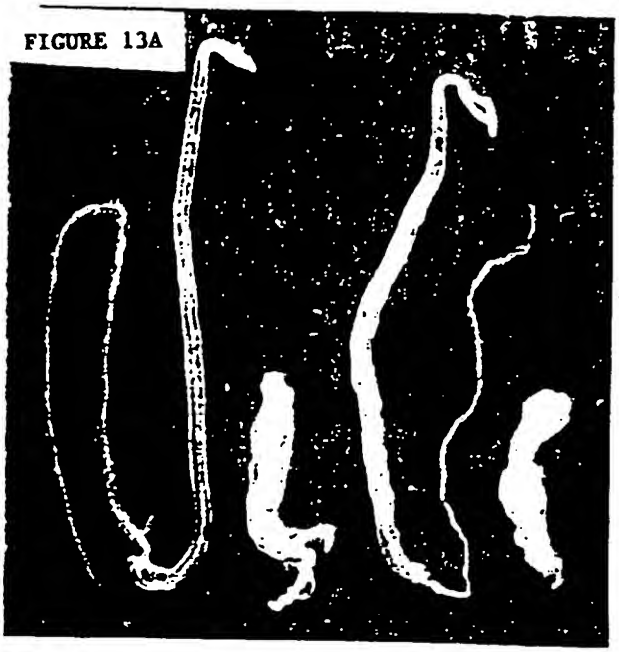


FIGURE 13B

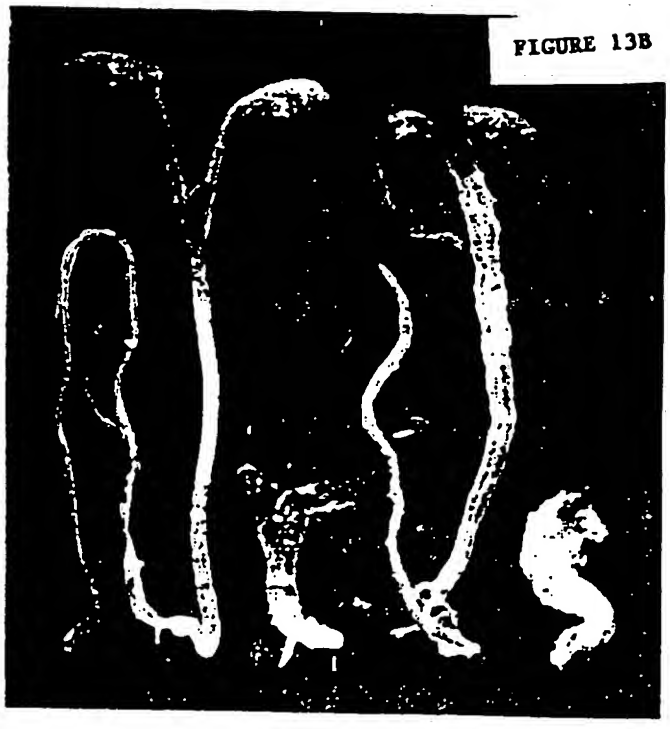


FIGURE 14 / SHEET 1

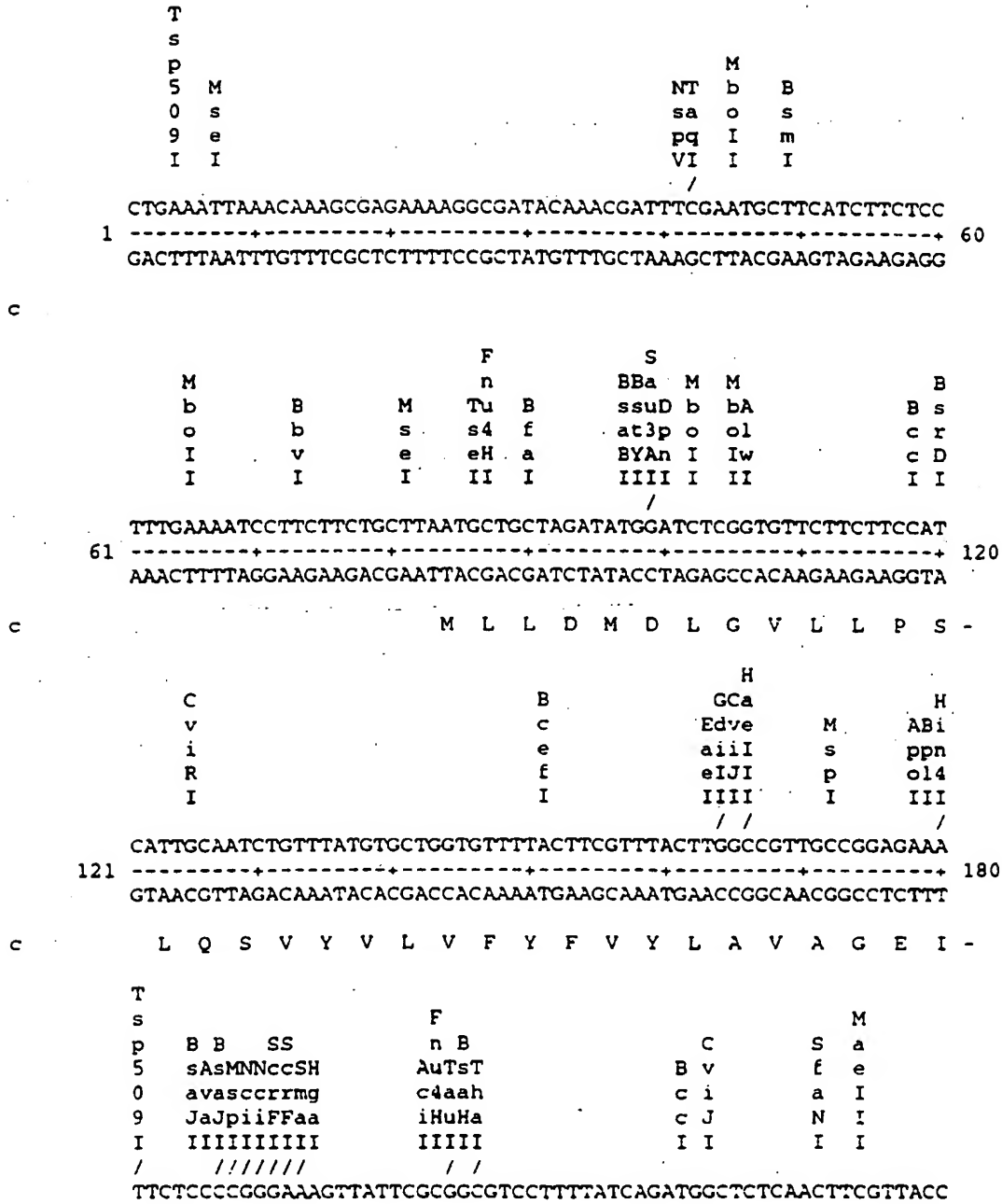


FIGURE 14 / SHEET 2

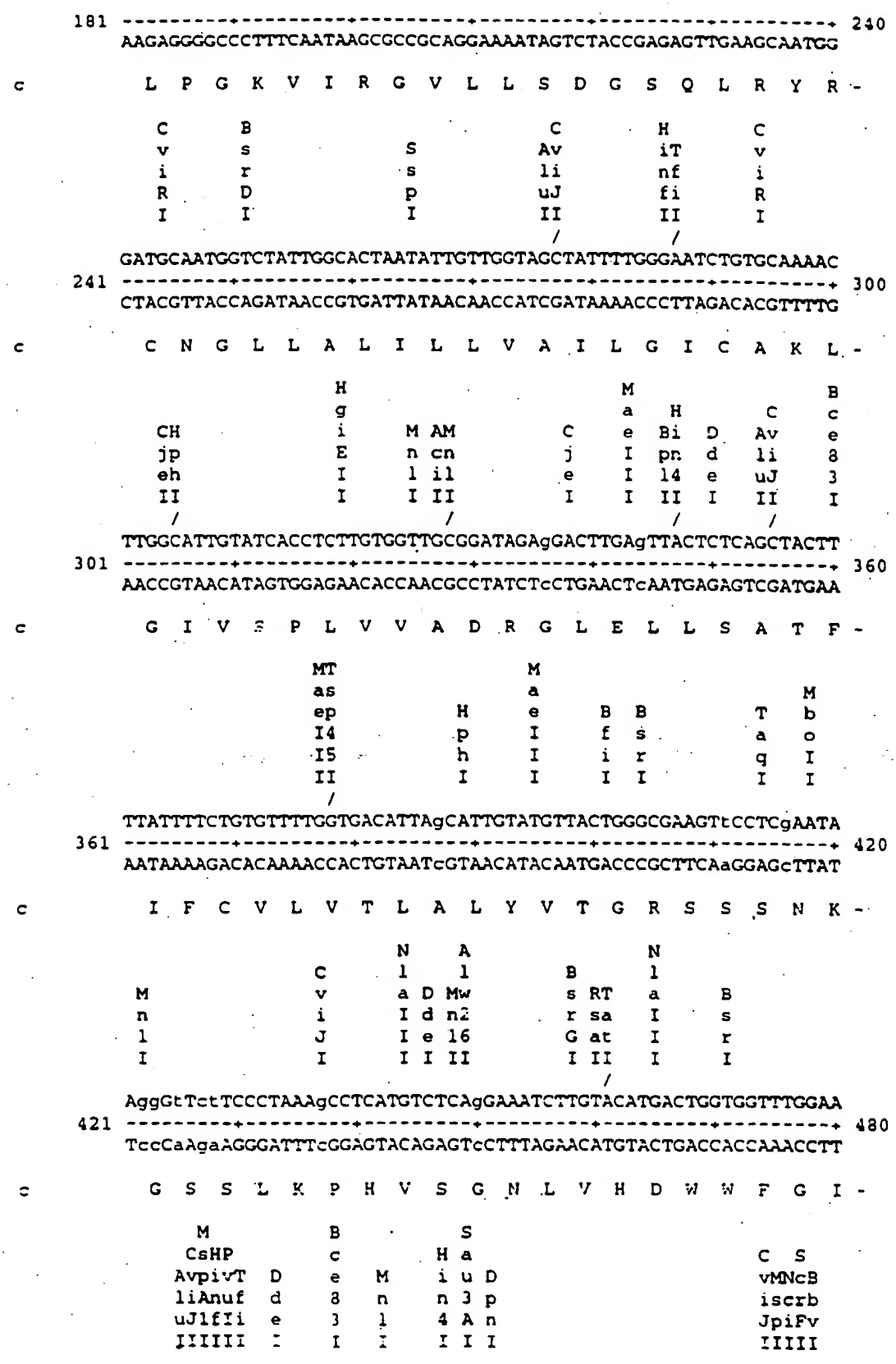


FIGURE 14 / SHEET 3

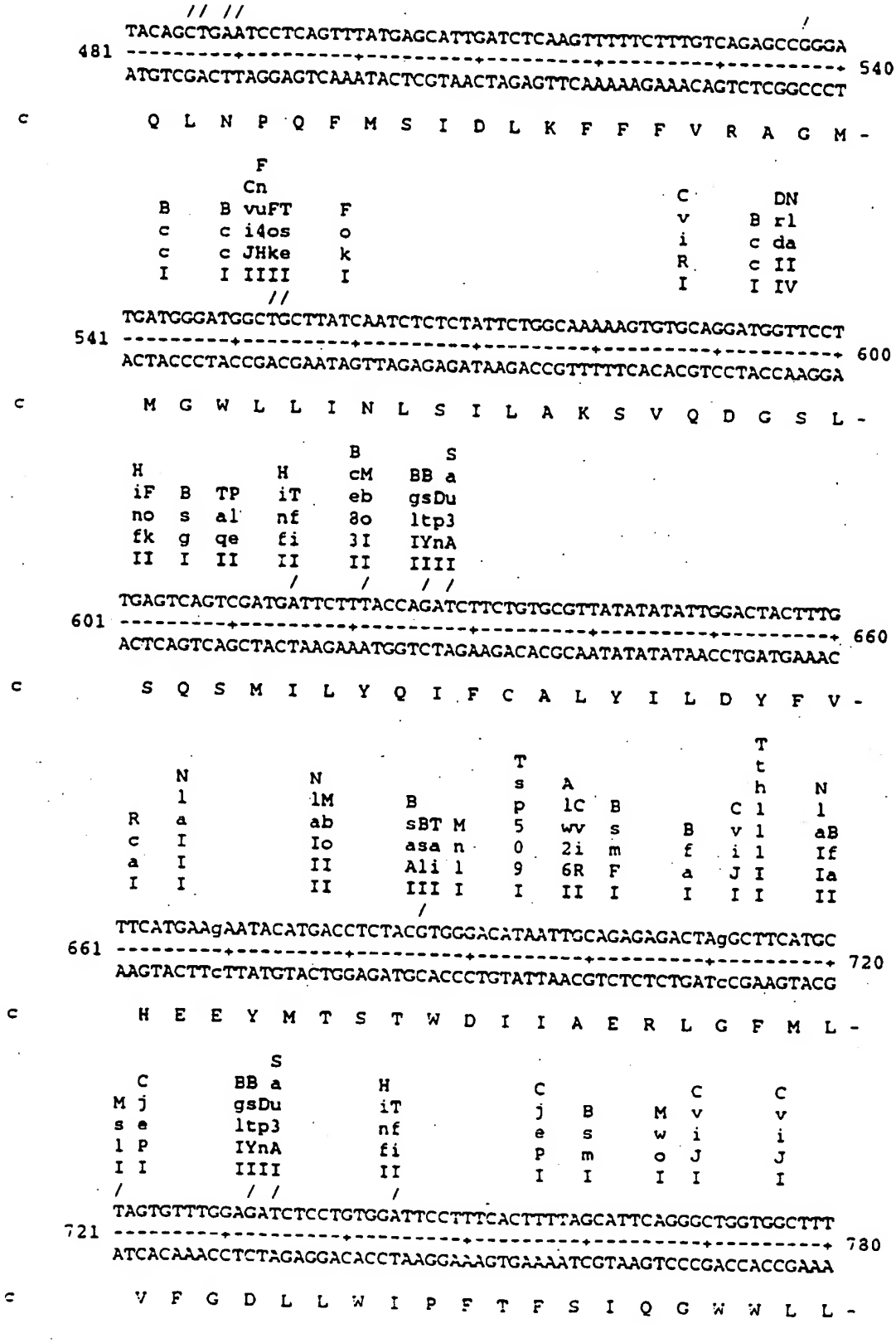


FIGURE 14 / SHEET 4

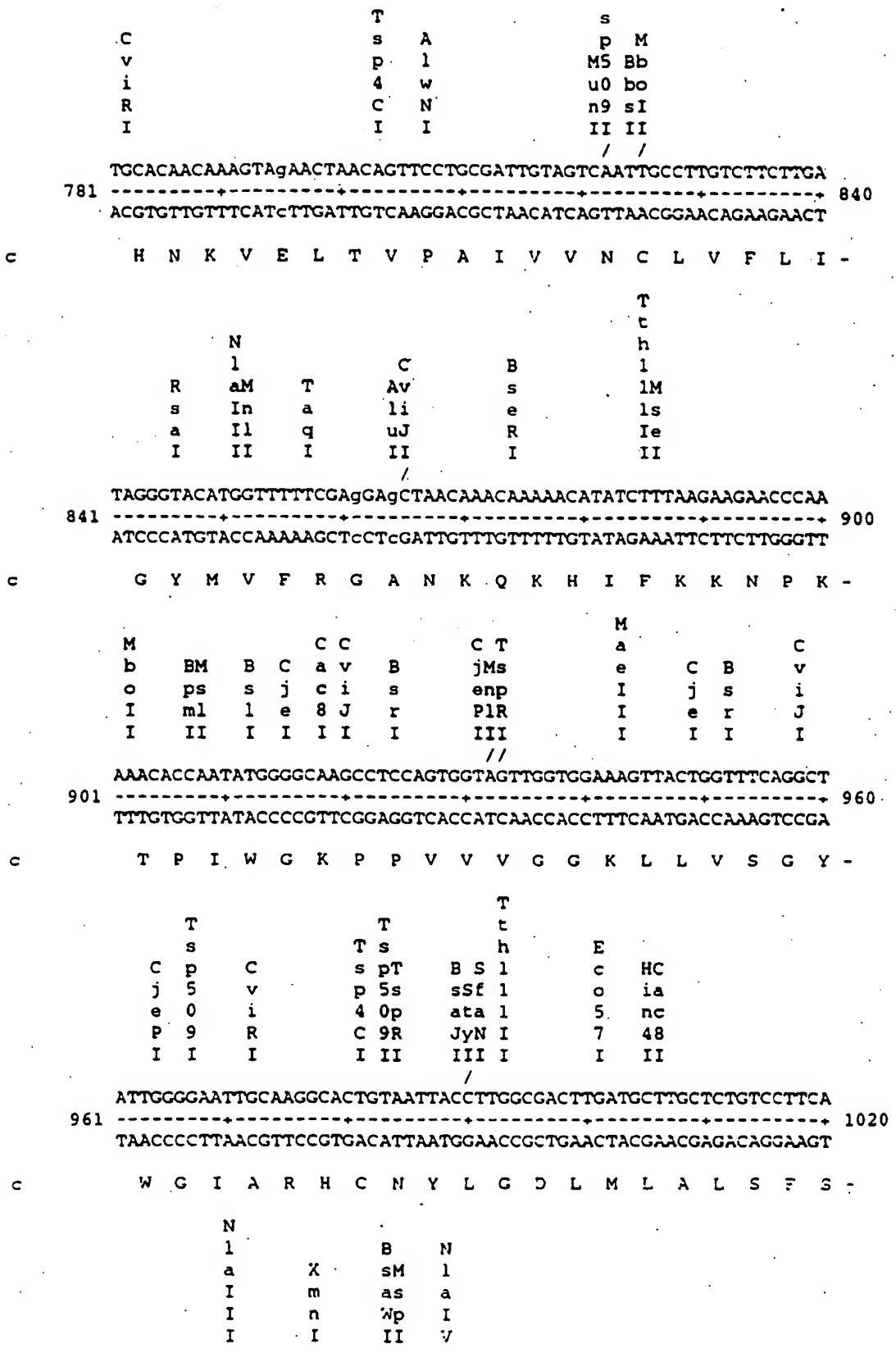


FIGURE 14 / SHEET 5

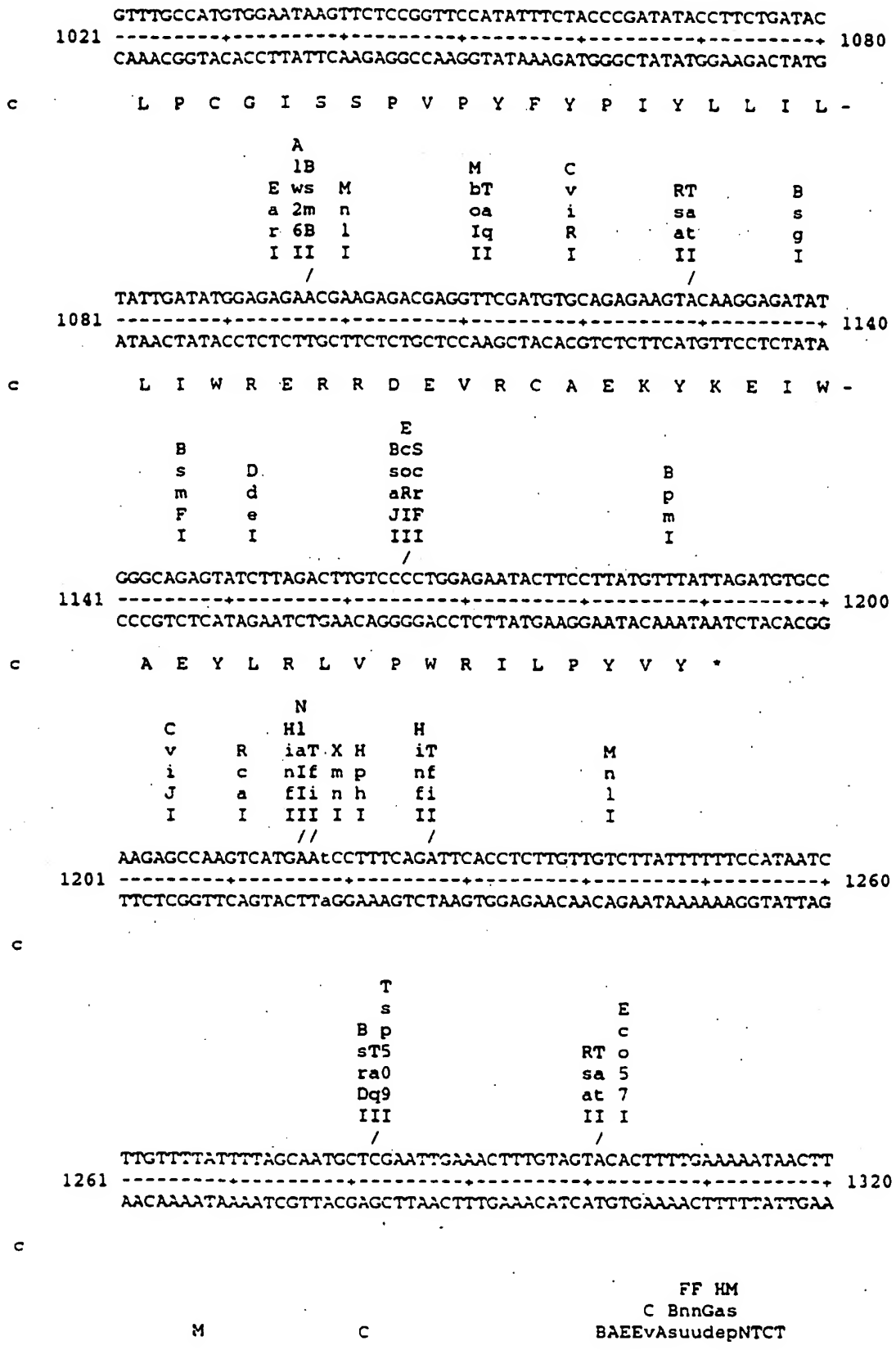


FIGURE 14 / SHEET 6

```
        s             j                        scaaici44iIAoaja
        e             e                        liegJiEHHIIltueu
        I             I                        IIIIIIIIIIIIIIII
                                                 /  //////// /
     CAGTCCTTAAAAAAAAAAAAAAACCTAANTTACTCCCNCTGGGCGGCCGCTGGTTTTATAT
1321 ---------+---------+---------+---------+---------+---------+ 1380
     GTCAGGAATTTTTTTTTTTTTTTGGATTNAATGAGGGNGACCCGCCGGCGACCAAAATATA

c

          T     T           S
          s     s           a
          p     p           u D
          5  M  5           3 p
          0  s  0           A n
          9  e  9           I I
          I  I  I
     TTGTTGTAAAAATTAAANAATTACTNCCTTGANGATCTGTAAAAAAAAA
1381 ---------+---------+---------+---------+--------- 1429
     AACAACATTTTTAATTTNTTAATGANGGAACTNCTAGACATTTTTTTTT

c
```

Enzymes that do cut:

AciI	AluI	AlwI	Alw26I	AlwNI	ApoI	AvaI	BbsI
BbvI	BccI	Bce83I	BcefI	BfaI	BfiI	BglII	BplI
BpmI	BsaAI	BsaBI	BsaHI	BsaJI	BsaWI	BseRI	BsgI
BsiEI	BslI	BsmI	BsmBI	BsmFI	BsrI	BsrDI	BsrGI
BstYI	Cac8I	CjeI	CjePI	CviJI	CviRI	DdeI	DpnI
DrdII	EaeI	EagI	EarI	Eco57I	EcoRII	Fnu4HI	FokI
GdiII	HaeIII	HgaI	HgiEII	Hin4I	HinfI	HphI	MaeIII
MboII	MnlI	MseI	MslI	MspI	MspA1I	MunI	MwoI
NciI	NlaIII	NlaIV	NotI	NspV	PleI	PvuII	RcaI
RsaI	Sau3AI	ScrFI	SfaNI	SmaI	SspI	StyI	TaiI
TaqI	TatI	TauI	TfiI	ThaI	TseI	Tsp45I	Tsp4CI
Tsp509I	TspRI	Tth111II	XmnI				

Enzymes that do not cut:

AatII	AccI	AceIII	AflII	AflIII	AhdI	ApaI	ApaBI
ApaLI	AscI	AvaII	AvrII	BaeI	BamHI	BanI	BanII
BcgI	BcgI	BclI	BglI	BmgI	Bpu10I	Bpu1102I	BsaI
BsaXI	BsbI	BscGI	BsiHKAI	Bsp24I	Bsp24I	Bsp1286I	BspEI
BspGI	BspLU11I	BspMI	BsrBI	BsrFI	BssHII	BssSI	Bst1107I
BstEII	BstXI	Bsu36I	ClaI	DraI	DraIII	DrdI	DsaI
EciI	Eco47III	EcoNI	EcoO109I	EcoRI	EcoRV	FauI	FseI
FspI	HaeI	HaeII	HhaI	HincII	HindIII	HpaI	KpnI
MluI	MmeI	MscI	NarI	NcoI	NdeI	NgoAIV	NheI
NruI	NsiI	NspI	PacI	Pfl1108I	PflMI	PinAI	PmeI
PmlI	PpuMI	PshAI	Psp1406I	PstI	PvuI	RleAI	RsrII
SacII	SalI	SanDI	SapI	Sau96I	ScaI	SexAI	SfcI
SfiI	SgfI	SgrAI	SimI	SnaBI	SpeI	SphI	SrfI
Sse8387I	Sse8647I	SstI	StuI	SunI	SwaI	TaqII	TaqII
Tth111I	UbaDI	VspI	XbaI	XcmI	XhoI		

FIGURE 15 / SHEET 1

```
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  51  TGTNACCACA ACACAACAAC GCCATGAACC NATCGGTTTC TTNTGTTTNG
 101  AGATCACCTT TCTTGAGTTG GTGGTTTCTG AGNTCAAGNT CCTTGTTGAC
 151  TCAGTGAAGT CCAGATGCAG CNTCAAAACT TTTGTCCTGT AGACNTAGCA
 201  AGAGTAACAG CACCAACCAA ATCGCTATCC GATGTAATCA AAACCTTATC
 251  ACNTTCATCG TCCTCATATA TAATCTGAGG CCGTTGTTCC ACATTGTTAT
 301  TGTCGCTGCC AATTCTTTGC ATCACAATAC CCATCAGCTN TTCGAGGTTT
 351  TCAGCTCCAG AAGTAAACCG ATGTACACGG CCCTTAAGGT CTTCAAATTT
 401  GAACGAAAAC GAATTCCCTA GTCCTAGAGA TGGGTAAGAA CTGAGCTTCC
 451  CTATATCTGA ATGATGCATC ATTGCCGACA TTTCACTTTG AGTGTCAGAA
 501  TCATCAGGTG GCTCTAACGC AAGAGCTGAA TCCCAAAATT TCTGCATCAT
 551  CGTGTTTGCC ATATCATTTA CAGCTCCAGA ACTGTTCTCC ACCATTGAAA
 601  TAGCTGCGTG AGTAATCTGA AGAACGTCTA CACAAGCTGC AGCTGATCCA
 651  TCTTTATCTA TAATTGGAAG ATGTAGAAAC TTTCCATCAT GCATTGTATG
 701  CAATGCATCC AGAATCGTTG TCTCTAGCGA TGCACATTCA GGATTCGGTG
 751  TCATTACCTT CTCGACAAGA GTCAATTCAG GAGATAAATT TTGTGCCACC
 801  ACTCGCATCA GAATGTCCTT TGAAGTCAAG ATTCCACTGA TTTTGTTCCC
 851  CCGTGGAAAT GATTACAGAG TTAACCCGCA AATCCCTCAT CCTTTTCGCA
 901  GCAACTGAAA CAGGATCTGA TGGTGCTACA AGTGCAACCT TCGATGTCTG
 951  TAATAATCGT GACAAGGCGG GTTAAACATT NTNTCCTTCA AGGTTTCAAT
1001  GAAAGCATAC GGTGCAGAAT ATCCGCTTCC CCATTGTTTN TCCACACCTT
1051  CCACTGCAGC AGCTAAAGCA CTACCTTGCT CTGCAGTTTC TCCATCCTAG
1101  AAATAGCATC ATACAAACAC TTTGTAATAT CCAACAAAGC AATGACTTCA
```

FIGURE 15 / SHEET 2

1151 CCATTCTCCA CAACAGGCAA GTGTCTAAAC TTCCCTTGAA CCATCTTCTG

1201 AAGAGCCTCA AGCGCCAACG AATCAGAAGT AACAAAAATA GGATTCCTAG

1251 TCATAACCTT AGAGACCAAA GTTTGATCCG GTCTCAACCC TTCAGCAATC

1301 ACTCTTGTAG CTACATCTTT ATCAGTAACA ATCCCGGAAA GAAGCGCACT

1351 TGAATCAGTC AACAAACAAG CATCAACACG CCTAGCAGCC ATTNTTCGAC

1401 AAGCATCGAA AACAGTAGTT CCTTCNAGGA ATAGTAAGAG CTTTCGATAA

1451 CCTAAGCTTC TTCGCTGTCT CTCTCCATTA GAAGGAGCTT GAGATTGAGG

1501 TTGAGGAGGA GGTGAATTGG GTTTTGAGGT GTTCCCANTA ACACTTCCAT

1551 TCTCTGATTG TACTGGTTTC TTAGAAGGTG GTGGTCCTCN CCGTACAGTA

1601 GAATTGCTTC TCCTCCCTGA TGTTGAAGAA GGACCCGTCG CTTGAGTACT

1651 CATATTCGGT CAATCTAGGG TTTACTTAGA TCCTAAATCC GTCANAAATG

1701 ATTCCTTTAG ATATCAAACT CGTCTCTGCA AATGAAAAAT TCAACCTTTA

1751 ATTCACAAAC TATTGAAATT TCATCTAAAG CACGAATCTG AATAAAACCC

1801 AATTCACAAT AAAGACGATT TGCTCTGAGA ATACGATGCA ACATACACGA

1851 AAAGGATTCG AATTTAACGG ACGAGGGAAA TGAAACAACT TGAAACCCTA

1901 AGGATTTGAG CAGAAGTTAT GTGGGAAGAT TGGGNATTTA GGGTTTACCT

1951 TCTTCTTTCT TCNTCAAGGT CTCTCTCTCG AGCACTTTCG TTNCCCCAAA

2001 AACNAACGGC TCTTAACAAT TGAGTTAANC CANTTATCGA GTTTTCATTG

2051 GNTGTTCCTG TTTCCGCGTG TGTGGTGGNT CNCCACCTCC TTTCTTATAA

2101 TCNACGACTA AAAATGTTAA ANATAANACT AANATTTCTT TCTANAAAAA

2151 TCGTAAAANC CAAATGTTTT TTTTTTTCTG ATAAATGTCT ATAAATCACC

2201 CTTTCTTTTT AAATAATGAA ATTTGATGAC ATTTATCTCT TGTATCTAGN

2251 AGAGTTAATG GCTAACATAA ANACCAAAAA AAATTAATTC NAATAAATAT

2301 GATTTGTGTG GGTTACATGG AAAAATTGTC AAATAATAAA NCAAAAAAAA

2351 ATTGTATAGA TGCAGTGCAA GTTGTTTCTG GTCAACTTGC CGTCGAGCCT

2401 CACAACTGTT TGTTACAAGT GGACTCGCAT GTAATTCCCT CTTTTAATAA

2451 CTTACCAGTT ACACCATCCA ACATGTGATT TGACAGAAAA ATATTTTAGT

2501 GAAATGTGAT CGGTGCAGAT TTTTCTATGT ACGTTTAAGC CTTTAAGGTA

2551 GACGTTTAAT CCNAAAATAT. CCCTGAATAA CAACACCGAT TAATGGAACC

2601 AAGTAGATAC CTCCTCCGTT TGGATGGCTC AAATGCAACC ATGATGCAAG

2651 CTTTTGCGAT TGACCCAAAG TGAGAGAACT AGATCGAGAT GGATTATTCG

FIGURE 15 / SHEET 3

2701 GAACCATTAC GGCACCCTTA TATAATGGCA GCATCTTAAT AGTAAACAAA

2751 AGCTTTAGCC TTAGGTTTTA GCTTCCTTCA CTCTTTGCAT ACATTGTGAA

2801 TCTGCGGTTT TAGATGGACC ATAGTGGAAA AAGGCTTTCA TCAATAACTC

2851 GTGGACTTGA TCAATGGTAG AAAAGANAAT ACATAGTATG GAAAACTAGA

2901 TATTTGATAT ATTTGGTTCA AACTCTTATC CGGTGTTGAG GTGATATACA

2951 CATGAAGACA TAACAATCGC ATAGCCGAGA AACTAGTATT CATTAACCTT

3001 TTTCTCTAAA GAGATTGTCC TATCAATCTA AATTTTAGAT GTTAAAAAAA

3051 AAATggtaag gttaaacagg ccgctaggtt ggttttacga tgatgtaaaa

3101 agtagccatc ttaaaataac agtcgtttgc gagactggcc aggccatccc

3151 atgggccata ggctcgctca agttgTGCTT GGCAGAATTT AGTAACTTGG

3201 GGTTTTGTTA TCAACAATCA ATAGTTTAAG GCTTTACCTG CAAGAAATGA

3251 AGAGTTTAAG GGTTCTTTTT GGTATTCCCG ATTCACACAA GTGAGCTAGC

3301 TCATCAGAGT CCACGAGCTT CCCACTAAAA AATTGAAAAT TGTTGCTTCT

ELL

3351 GTCATCTGAA ATTAAACAAA GCGAGAAAAG GCGATACAAA CGATTTCGAA

3401 TGCTTCATCT TCTCCTTTGA AAATCCTTCT TCTGCTTAAT GCTGCTAGAT

3451 ATGGATCTCG GTGTTCTTCT TCCATCATTG CAATCTGTGA GCTGTCTCTT

3501 TAGCTTTTGA CTGTTGCAAT TGTTATTGTG AAATTTTTGT TCGCTTTTGG

3551 ATCAGCTTTT GTTAAATTCG TTCCGAGATT TTAGGTTTAT GTGCTGGTGT

3601 TTTACTTCGT TTACTTGGGN CGNTGGCGGA GAAATTCTCC CCGGGAAAGT

3651 TATTCGCGGC GTCCTTTTAT CAGATGGCTC TCAACTTCGT TACCGATGCA

3701 ATGGTATATT TGATTTGATT TACTCTCTCT ACAATTCCTG AGAGTCTGTG

3751 AGCTCGAAAG TTCATTTCCA TTAGTTTGGT TAATTCAATT TCAGGTCTAT

3801 TGGCACTAAT ATTGTTGGTA GCTATTTNGG GAATCTGTGC AAAACTTGGC

3851 ATTGTATCAC CTCTTGTAAG TGTAGTTACA AGATTTCGAT TGTATTTCTA

3901 TGAATCCGAA TGCTATATGC TATATGAATC CGATTGCAAT TGCTTTCTCA

3951 CACTCATTCC ACTGAGATGT TTGGTAGGTG GTTGCGGATA GAGGACTTGA

4001 GTTACTCTCA GCTACTTNNA TTTCTTGTGT TTGGGGAAGA TGATCAATCC

4051 TTAGTCCGGN GTCTTGGATT TTAGNTGNGT TACCATCAGA TTNGCTTTGG

4101 GTGGTGTGAT TTGTAATCTC CATGATATCT CTTAATATTC TCAGGTGACA

4151 TTAGCATTGT ATGTTACTGG GCGAAGTTCC TCGAATAAGG GTTCTTCCCT

4201 AAAGCCTCAT GTCTCAGGAA ATCTTGTACA TGACTGGTAC TAACATAATA

4251 CAATTGTAGA TCTGATACTT TCTTGTTACA CAAAATGTTG TTAAAAGTTA

4301 TATATTTTGA CTCCTGCAAG AGCAAAACTA AGAAATAATC TGGTACTATA

4351 TAGAGTTTGA AACACTGAAT TGGACAAGAT GATTCTATAG AACTTCGTAG

4401 AGTGTTGAGT AATTTCTCCT AGAACGGTTG TAGCTTCCTC TTTTTTCCTT

4451 TTAACCGCAG TGACTTTAGC TTTTGGAACT TTTCTACTGA AACTAGAAGT

4501 TCTGGTTTTG TCTTTCACTT ATCTCTTCCA AACAACTGCT TCAATTTTTT

4551 CTCATATTGT TTGTTTCATG TGATAGGTGG TTTGGAATAC AGCTGAATCC

4601 TCAGTTTATG AGCATTGATC TCAAGTAATC CATTTTTCTG TTTTTTCTTC

4651 TATTTGTCAG CCAAGGCTAC ATCATTGCTT CAGTTTGTTC CGTACTCAAT

4701 CGAGTGGCAG TTTAATAATG TAATCAGCAG TTATGCATGG TTATGATGAA

4751 TGGGAGTTAT TCCTTGTGTA GGTTTTTCTT TGTCAGAGCC GGGATGATGG

4801 GATGGCTGCT TATCAATCTC TCTATTCTGG CAAAAAGTGT GCAGGATGGT

4851 TCCTTGAGTC AGTCGATGAT CTTTACCAGA TCTTCTGTGC GGTAAATTTG

4901 GTTTTTACTT ACAAATCTTG CTTCTTGAAN TCTGATCATC TGTGTTTTGT

4951 TAGTTTTGAT TAGTTTTATA ATTGCAGTTA TATATATTGG ATACTTTGTT

5001 CATGAAGAAT ACATGACCTC TACGTAAGTT CATGGCGTGT TAAGGAAACA

5051 CATTTGTCTT ACCAAAAAAT GACCATTTGC ATTATTACAT CTACTTTGAT

5101 TTTACTCTTT TCAGGTGGGA CATAATTGCA GAGAGACTAG GCTTCATGCT

5151 AGTGTTTGGA GATCTCCTGT GGATTCCTTT CACTTTTAGC ATTCAGGCAT

5201 GTAACTGTGA GCCTGAACAC AAACAAGATA TTAATTTATC TTATTGACAG

5251 TATCTTCTTG GCATGTTACA GTTATTCTCG GAAACAATAT TGTTCTAGAA

5301 TGCTTGATCA CTCTGTGACT GAATTGTCTT CTCTCTGGTA CAGGGCTGGT

5351 GGCTTTTGCA CAACAAAGTA GAACTAACAA TTCCTGCGAT TGTAGTCAAT

5401 TGCCTTGTCT TCTTGATAGG GTAAGTTCTG AGACATGGGG TTATTTTCCA

5451 TTCTTACATA TCTACACTAA GAAACCCACT ATTTCTTCTT TGGCAGGTAC

5501 ATGGTTTTTC GAGGAGCTAA CAAACAAAAA CATATCTTTA AGAAGAACCC

5551 AAAAACACCA ATATGGGGCA AGCCTCCAGT GGTAGTTGGT GGAAAGTTAC

5601 TGGTTTCAGG CTATTGGTAT GTTATATTTA TCTTCTCTTG TTTCTTTGCT

5651 TGGTTTCGCC ATCTCTGTGT TTGATTGTTC ATCATGCTGG GAATAAAGAG

5701 TTGAAAGTTC CGCAATGACA CATTTCCGAT AACTTAGGTG CTGTTTTGTA

5751 TATATGACAG GGGAATTGCA AGGCACTGTA ATTACCTTGG CGACTTGATG

FIGURE 15 / SHEET 5

5801 CTTGCTCTGT CCTTCAGTTT GCCATGTGGA ATAAGGTACT CCTNCTGCTT

5851 GAGTTCACTT ACAGCTACCA AAATCATGTA GAAACTAATA CCAATATCNA

5901 AACGTTCGAA GTTGATTTGG CTGACTTAAA GATATTGATC TCTAACCATC

5951 ATTTGAAAAG TCTAAAGCTT TCAAGTTCAT TTCCCAAAGC TGTTTTTATG

6001 ATATTTCGTC TNGTGTATTC TCAGTTCTCC GGTTCCATAT TTCTACCCGA

6051 TATACCTGCT GATACTATTG ATATGGAGAG AACGAAGAGA CGAAGTTCGA

6101 TGTGCAGAGA AGTACNAGGA GATATGGGCA GAGTATCTTA GACTTGTCCC

6151 CTGGAGAATA CTTCCTTATG TTTATTAGAT GTGCCAAGAG CCAATTCATG

6201 AATCCTTTCA GATTCATCCT CTTGTGTCTT ATTTTTTCAT TAAATGTGAC

6251 NTGAAATGAT CCCATTATNG CCTNTTATCA ATGCTTGATT GAAACTTTGT

ELL

6301 AGTACACGTT TGAGAATTAC TTCAGTCCTT|GTTATTATTT TAGCATGGAT

6351 ATCAACATTT TCGGATTTAT TTNTNGGGTT ATTTTAAAAC CNNAGATTAC

6401 CNAANAAAAC CATTGTTTGA NGTANGATAA TATGGACTTT TTACTGAAAA

6451 AAAATNCTAN TAGGGGAACA AATNGAAGTT GAATATGGCT GAATNTTTTT

6501 ATGGANAAAA TGGAAACTTT TCCCACTTTG AAATGACAAT NCAAGTTTGG

6551 TGGACNACTT AATCACTGGA AACGTTAATG GCCAACCN

FIGURE 16
SHEET 1

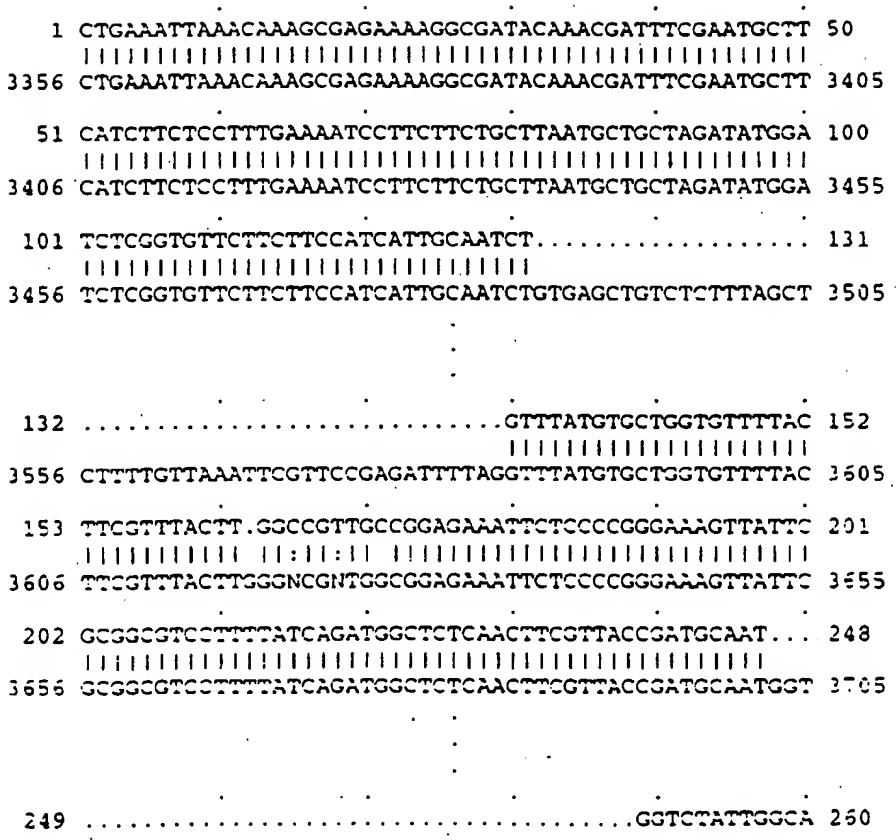


FIGURE 16 / SHEET 2

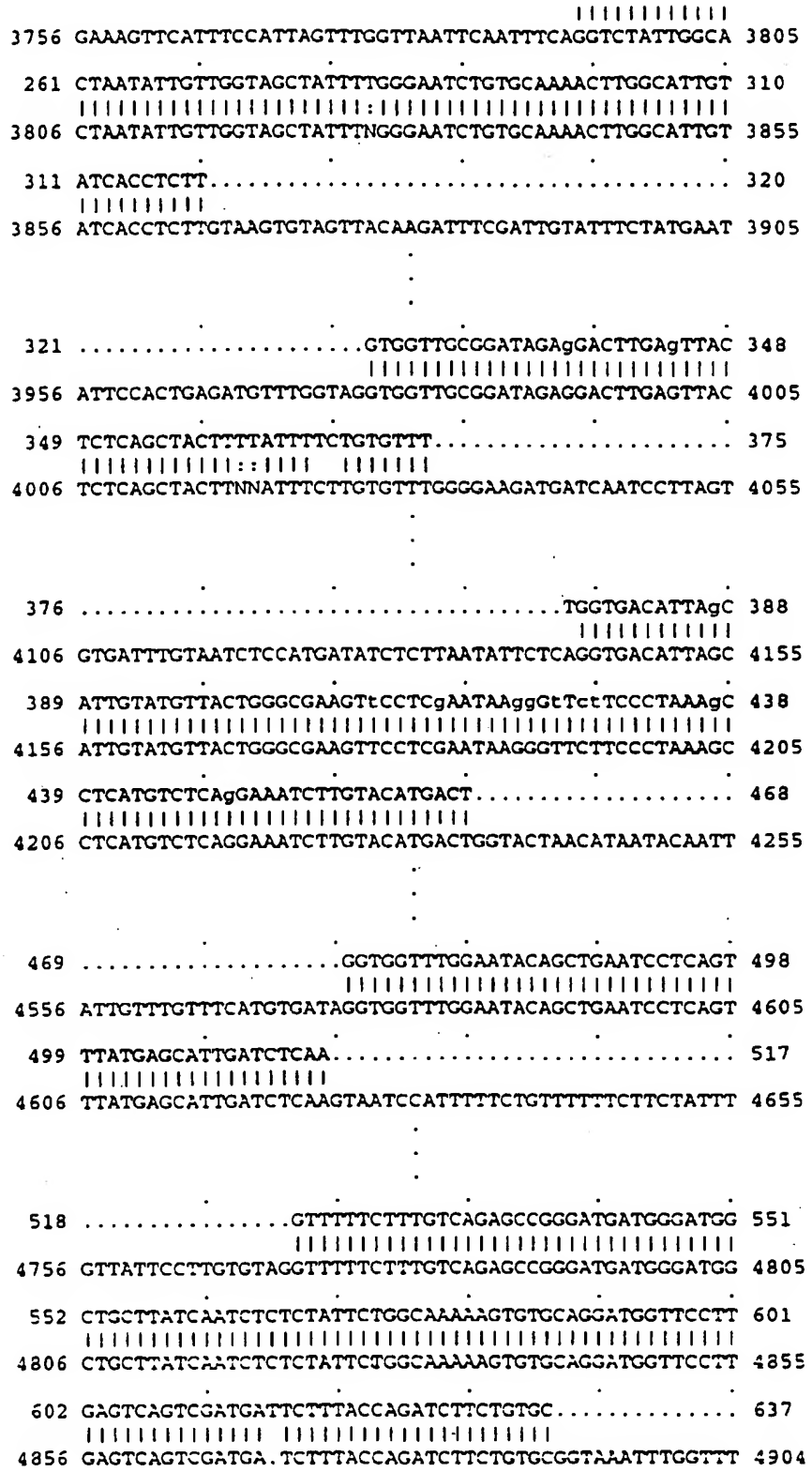


FIGURE 16 / SHEET 3

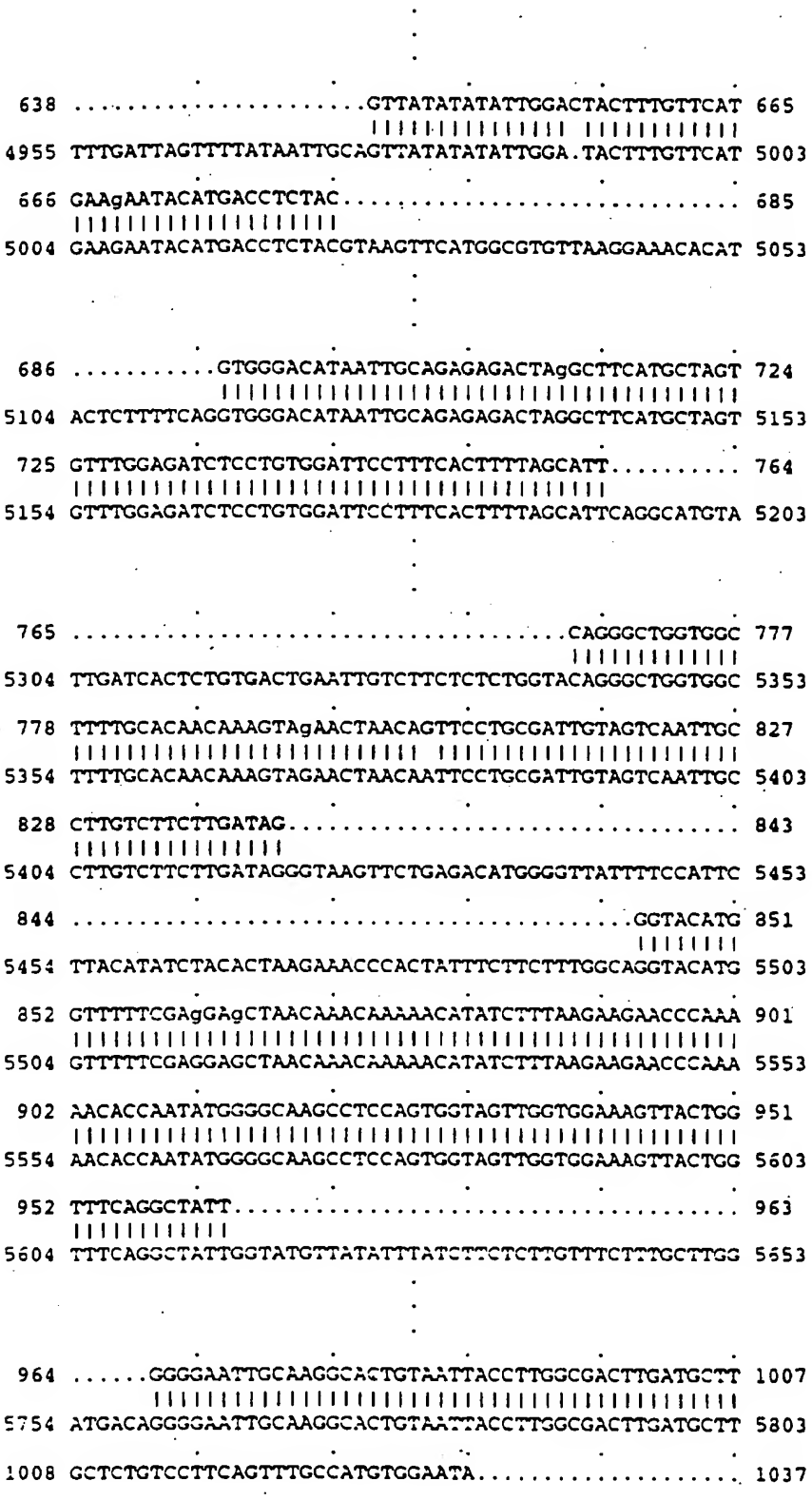
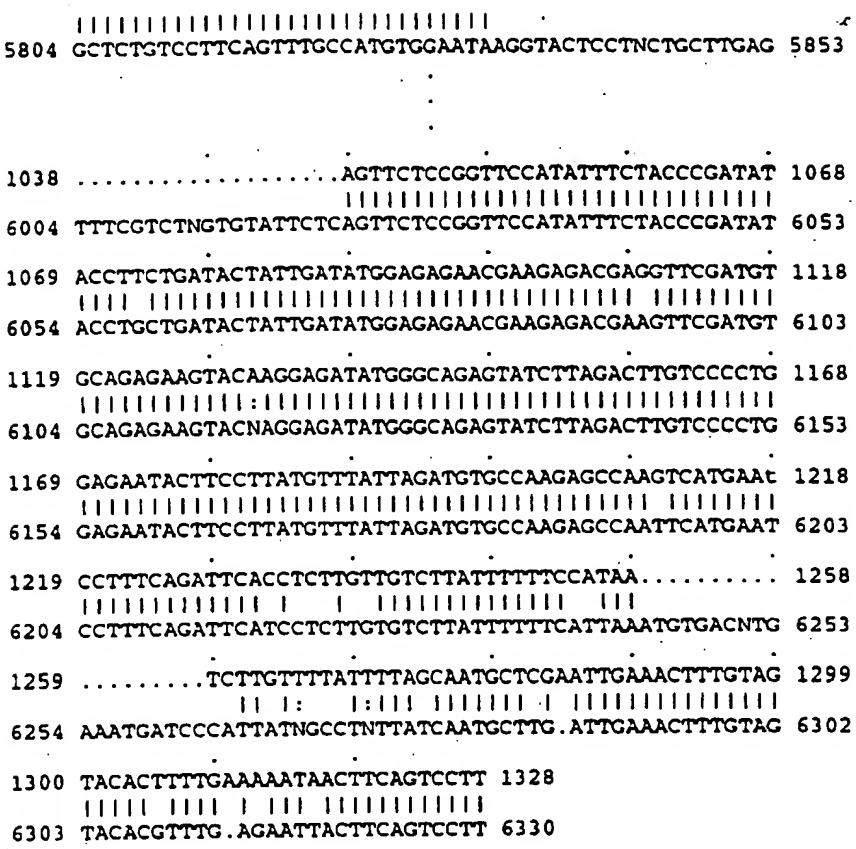


FIGURE 16 / SHEET 4



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/10644

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 9/02, 15/53, 15/63, 1/21, 15/09, 15/10; C12Q 1/68, 1/26
US CL :526/23.2, 23.6; 435/189, 252.3,320.1, 419, 6, 91.2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 526/23.2, 23.6; 435/189, 252.3,320.1, 419, 6, 91.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, SCISEARCH, LIFESCI, BIOTECHDS, BIOSIS, EMBASE, CAPLUS, NTIS, WPI, CABA, AGRICOLA
search terms: sterol reductase#

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TATON M. Microsomal $\Delta^{8,14}$-Sterol Δ^{14}-Reductase in Higher Plants. Eur. J. Biochem. November 1989. Vol. 185. pages 605-614. see entire document.	1-18, 24, 25
Y	GOAD L.J. --Application of Sterol Synthesis Inhibitors to Investigate the Sterol Requirements of Protozoa and Plants. Biochem Soc. Trans. 1990. Vol. 18 pages 63-65. see entire document.	1-18, 24, 25
A	LAI, M.H. The Identification of a Gene family in the Saccharomyces cerevisiae Ergosterol Biosynthesis Pathway. Gene 1994. Vol. 140. pages 41-49.	1-18, 24, 25

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 20 AUGUST 1997	Date of mailing of the international search report 0 4 SEP 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer REBECCA PROUTY Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/10644

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-18, 24 and 25

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/10644

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-18, 24 and 25, drawn to C-14 sterolreductase, DNA therefore, and methods of producing C-14 sterol reductase.
Group II, claim 19, drawn to C-14 sterol reductase antibodies.
Group III, claims 20-23, 30 and 31, drawn to transgenic plants.
Group IV, claims 26-29, drawn to a method for reducing C-14 sterol reductase expression.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The inventions of Groups I-III comprise different products with unrelated chemical structures. The method of group IV lacks a corresponding special technical feature because it neither makes nor uses the products of any of Groups I-III.